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A UNIFIED THEORY FOR THE INTERACTION BETWEEN MYOSIN AND ADENOSINE TRIPHOSPHATE^{*)}

By

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1. Reaction Mechanism

The first subject of this article is the reaction mechanism of the myosin-ATP system, especially phosphorylation of myosin by ATP. Phosphorylation of myosin by ATP has repeatedly been suggested, since it was first proposed by KALCKAR. But previous studies do not seem to have given conclusive results, and further studies are needed to show that myosin is actually phosphorylated by ATP. Our own work on the phosphorylation of myosin was started by KITAGAWA and the author, and has been developed by KUBO, KANAZAWA, IMAMURA, TOKIWA, NAKAMURA, KINOSHITA and others. Recently, we have obtained sufficient evidence to show that myosin is indeed phosphorylated by ATP and that this phosphorylation is a key reaction in muscle contraction.

We measured the time-course of P_i liberation from the myosin-ATP system, when the reaction was stopped by adding trichloroacetic acid. In our earlier experiments the concentration of myosin was usually 5 mg per ml and the amount of P_i liberated was measured by the Martin-Doty method.^{1,2)} Using γ - P^{32} labelled ATP, we found that the liberated phosphate was derived from the terminal phosphate group of ATP in the initial rapid phase of the reaction just as in the steady state. The amount of the initial burst, that is,

*) This article is not a review in the usual sense. Its purpose is to review only our own studies of this interesting but complicated reaction, and to present a unified theory for the molecular mechanism of the reactions, in which myosin, F-actin and ATP participate. Therefore, only our own references are included. Original work in the author's laboratory has been supported by grants from the Ministry of Education of Japan, Toyo Rayon Science Foundation, the National Institutes of Health, U.S.A., and the Muscular Dystrophy Associations of America.

the amount of P_i liberated in excess in the initial phase, was actually one mole per 4×10^5 g of myosin. This was true over wide ranges of KCl concentration, pH, and temperature, if the Mg^{++} concentration was higher than 1 mM. Later, we measured the amount of the initial burst at much lower concentrations of myosin (~ 0.1 mg/ml) than we had used in earlier experiments by using γ - P^{32} -ATP.³⁾ We again obtained the same value as in the earlier experiments.

There is much chemical evidence indicating that one mole of active site is present per $4-5 \times 10^5$ g of myosin. Recently, from sedimentation equilibrium and osmotic pressure measurements we found that the molecular weight of myosin is about 4.8×10^5 .⁴⁾ Cardiac myosin also showed one mole of rapid initial P_i liberation per mole.⁵⁾ The amount of the initial burst of P_i liberation from H-meromyosin which was isolated from myosin digests with trypsin, was almost equal to one mole per mole of protein.⁶⁾ The amount of extra P_i liberation increased linearly with the ATP concentration until the amount of ATP reached one mole per 4×10^5 g of myosin.²⁾ At higher ATP concentrations than this, the amount did not increase further. This seems to show that one mole of P_i is liberated per mole of active site of myosin in the initial reaction. The initial rapid liberation of P_i was inhibited by blocking the sulfhydryl groups of myosin with PCMB.^{2,7)} The initial rapid liberation was also prevented by removal of free Mg^{++} from the reaction mixture by adding EDTA or by exhaustive dialysis.^{2,7)}

There are several possible mechanisms for the stoichiometric liberation of P_i in the initial phase. The most likely seems to be that the extra P_i liberation is caused by cleavage by trichloroacetic acid of an intermediate which is phosphorylated at a carboxyl group of myosin.⁸⁾ This view is based on the requirements for Mg^{++} and sulfhydryl groups and the apparent high instability of the supposed intermediate in the presence of trichloroacetic acid.

We tested this possibility by following the liberation of hydrogen ion in the ATPase reaction at about pH 8.^{8,9)} Hydrolysis of ATP at pH 8 must be accompanied by liberation of one mole of hydrogen ion per mole of ATP hydrolyzed, whereas phosphorylation of a carboxyl group does not cause any hydrogen ion liberation. The change in hydrogen ion concentration was followed by the stopped flow method by measuring the change in absorption of cresol red at 590 m μ .⁹⁾ Fig. 1 shows the time-course of the change in hydrogen ion concentration after the addition of ATP to myosin. We found that about one mole of hydrogen ion per 4×10^5 g of myosin was rapidly absorbed immediately after the addition of ATP. The absorbed hydrogen ion was then released again in a time roughly corresponding to the time required

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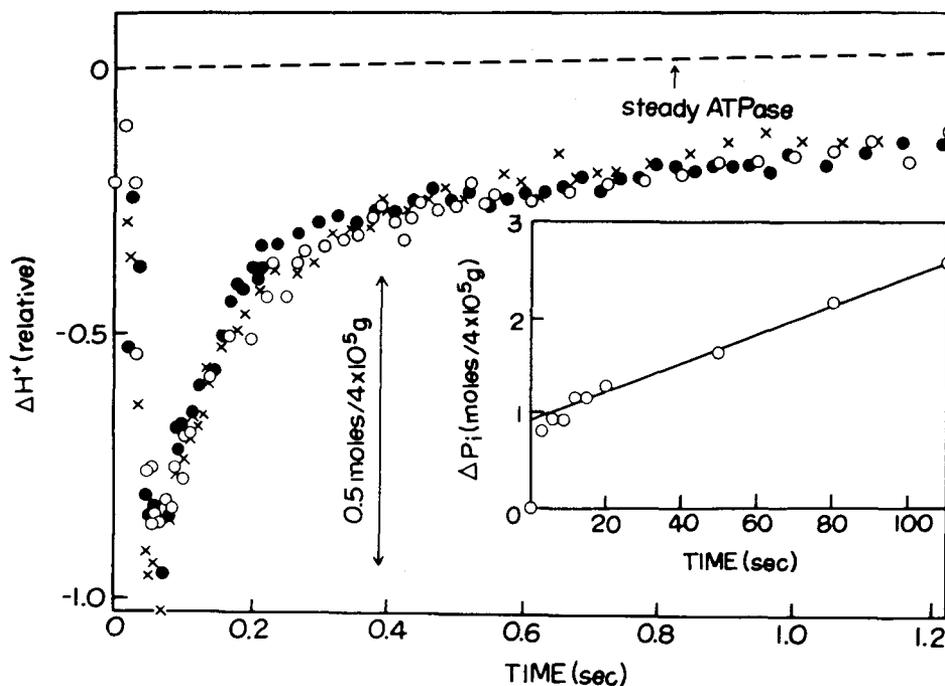


Fig. 1. Time-courses of rapid absorption-liberation of hydrogen ion from myosin-ATP system.

2 mg myosin/ml, 1 mM ATP, 1 M KCl, 1 mM MgCl₂, pH 8.2, 24°C. ○, ●, × : three independent records of the change in H⁺. The inserted figure shows the time-course of liberation of P_i of the same myosin preparation under the same conditions.

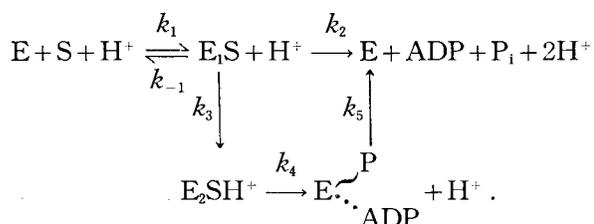
for the initial burst of P_i liberation. After the initial rapid absorption and liberation of hydrogen ion, a slow liberation of hydrogen ion continued to take place at a rate corresponding to that of steady state hydrolysis of ATP. Therefore, the overall reaction in the initial phase was not accompanied by hydrogen ion liberation.^{8,9)} Thus, this result supports the idea that the phosphorylation of a carboxyl group of myosin with ATP is the initial reaction. Furthermore, our studies on the effects of several modifications of myosin on the myosin-ATP system clearly showed that the same mechanism was responsible for both the rapid absorption and liberation of hydrogen ion and the initial rapid liberation of P_i.⁹⁾

We have also measured the liberation of ADP from the myosin-ATP system, by measuring the oxidation of NADH in the presence of phosphoenol pyruvate, pyruvate kinase and lactate dehydrogenase.⁶⁾ It seems likely that only free ADP is available to the kinase system and nor ADP bound to

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myosin. If so, the dependence of the rate of oxidation of NADH on the ATP concentration in the steady state in this system could easily be explained by supposing that myosin contains one mole of ATP-binding site per 4×10^5 g and that in the steady state myosin contains no bound ADP, but it could not be explained by supposing that myosin contains bound ADP. However, the rate of oxidation of NADH showed no initial burst.⁶⁾ Therefore, in the initial phase ADP as well as P may bind to myosin.*)

Accordingly, the above results can be explained by the following equation :



In this scheme the k 's represent the rate constants for the indicated steps. Here k_3 is larger than k_4 , and k_4 is much larger than k_2 and k_5 . E_1S and E_2SH^+ represent two intermediates of the reaction. According to the above considerations, the formation of EP is assumed to be due to the phosphorylation of a carboxyl group in moysin. In this scheme, two different routes are assumed for hydrolysis of ATP. This is because, as will be mentioned later, certain modifications of myosin, such as *p*-nitrothiophenylation, completely prevent the initial rapid liberation of P_1 , without affecting the steady state ATPase activity. As will be mentioned later, this idea that myosin ATPase is a double headed enzyme has been verified by determining the life-time of EP.

The existence of the first intermediate of the reaction, E_1S , was originally deduced from the fact that myosin-ATPase at the steady state follows Michaelis kinetics. Recently, MORITA has shown that the absorption band of H-meromyosin at about $280 \text{ m}\mu$ changes on the addition of ATP or ADP. She showed that this change is induced by the binding of one mole of ADP with one mole of H-meromyosin. We have measured the binding constants of ATP and ADP to H-meromyosin from the dependencies of the difference spectrum on the ATP and the ADP concentrations.¹¹⁾ The binding constant

*) When the concentration of Mg^{++} was much lower than 1 mM, the amount of the initial burst of P_1 liberation was more than 1 mole per 4×10^5 g of myosin.²⁾ In this case, the initial burst of P_1 liberation of 1 mole per 4×10^5 g of myosin did not accompany the liberation of free ADP but the remaining burst did.¹⁰⁾ This suggests a fundamental difference in the molecular mechanisms of these two types of initial burst.

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of ATP was almost equal to the Michaelis constant, and the binding constant of ADP was equal to the competitive inhibition constant of ADP of the ATPase reaction in the steady state. Thus, the difference spectrum is concluded to be due to the formation of E_1S . The difference spectrum was very similar to the red shift of the absorption band observed on a perturbation of the tyrosine residue, as suggested by MORITA. Actually, our studies on the interaction between synthetic ATP analogues and actomyosin have suggested the donation of the π -electron system of the adenine base to the benzene rings of the side chains of myosin.¹²⁾

To clarify the molecular structure of the intermediate, E_1S , we studied the effects of specific chemical modifications of myosin, as well as the kinetics of ATPase. We found that two moles of trinitrobenzenesulfonate bind to two moles of the ϵ -amino groups of the lysine residues in one mole of myosin.¹³⁾ In the steady state the Ca^{++} activated ATPase was inhibited, and the decomposition of EP in the presence of Mg^{++} was accelerated by this modification,

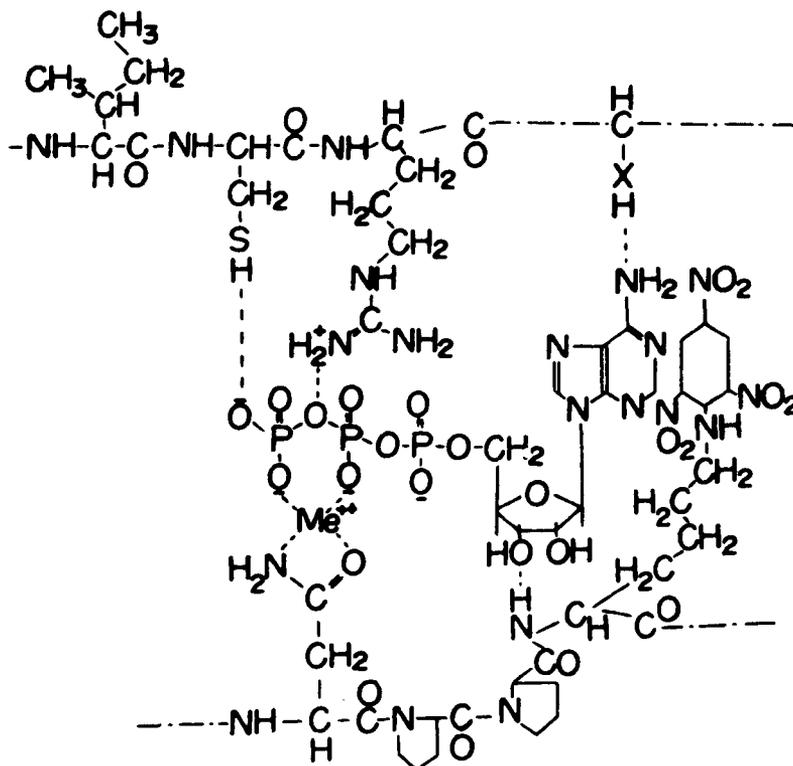


Fig. 2. A schematic model for binding of ATP with trinitrophenyl myosin.

as will be mentioned later. Recently, we found that the active fragment, Fr-2, of myosin contains only one mole of lysine residue per mole which is specific for binding trinitrobenzenesulfonate and that the chemical structure around this lysine residue is Asp(NH₂)-Pro-Pro-Lys.¹⁴⁻¹⁶⁾ We have also deduced the properties of the binding site of ATP with myosin by investigating the interaction of ATP analogues with actomyosin systems. Fig. 2 shows the structure of the ATP-trinitrophenyl myosin complex, deduced in these two ways.¹⁷⁾

The existence of the second intermediate, E₂SH⁺, was deduced from the initial rapid absorption and liberation of hydrogen ion in the myosin-ATP system, as mentioned above. In our previous analysis, we assumed that the rate of formation of E₂SH⁺ was much faster than that of conversion of E₂SH⁺ to EP and that the latter process was independent of ATP. However, during our studies on the exchange reaction, which will be described later, we observed that the rate of EP-formation is completely dependent on the ATP concentration. Furthermore, MORALES and his co-workers have recently reported that the change in the amount of hydrogen ion in the initial phase was less than one mole per mole of myosin, when a high concentration of ATP was added to myosin at a low KCl concentration.*) These two results prompted us to reinvestigate the rate of formation of the trichloroacetic acid labile myosin-phosphate complex and also the change in hydrogen ion concentration in the initial phase.

Fig. 3 shows the time-courses of P_i liberation after mixing myosin with various concentrations of ATP. The time-course of P_i liberation in the initial phase after adding trichloroacetic acid followed first order kinetics, the half-time of initial rapid P_i liberation was constant, and the amount was proportional to the amount of ATP, when the molar concentration of ATP was lower than that of myosin. But, when the molar concentration of ATP exceeded that of myosin, the rate increased with increase in the ATP concentration.³⁾ The initial rapid hydrogen ion liberation from the myosin-ATP system, measured by the stopped flow method, also followed first order kinetics. At low concentrations of ATP of up to 1 mole per mole of myosin, the amount of initial rapid liberation of hydrogen ion also increased linearly with the ATP concentration to about one mole per mole of myosin. As with the initial rapid P_i liberation, the rate of initial hydrogen ion liberation was constant when the molar concentration of ATP was lower than that of myosin. But, it increased with increase in ATP concentration at higher concentrations (Fig. 4). Thus, the conversion of E₂SH⁺ to EP seems to be accelerated by

*) Later, they have confirmed our result that the initial burst of H⁺-liberation is about 1 mole per mole of myosin.

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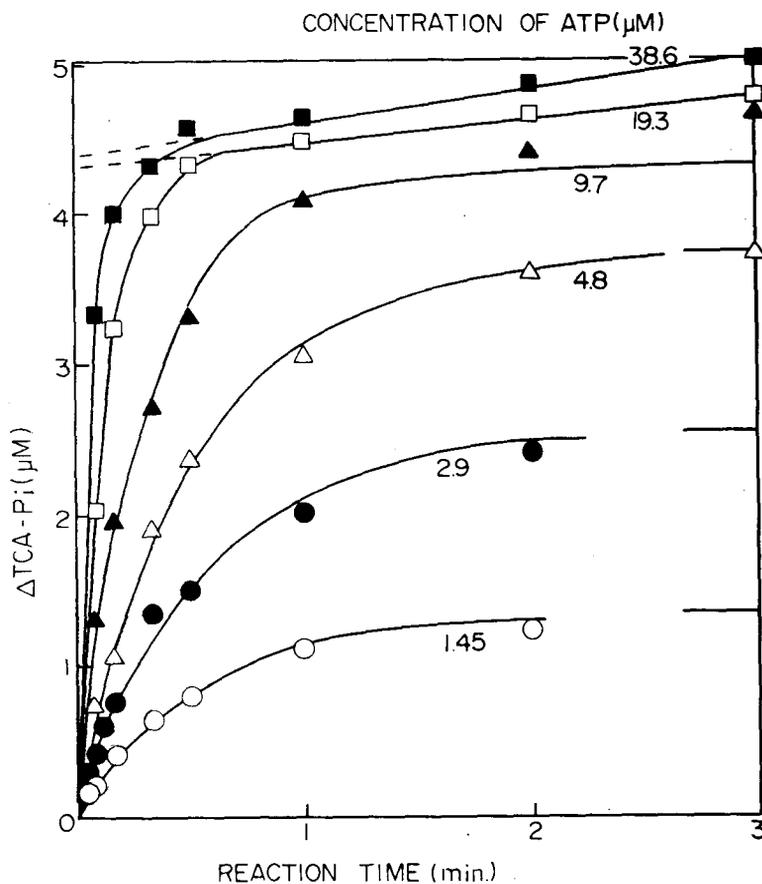


Fig. 3. Time-courses of P_i liberation in the initial phase after mixing myosin with various concentrations of ATP.

2 mg myosin/ml, 2.8 M KCl, 10 mM MgCl_2 , 20 mM Tris-maleate, pH 7.5, 0°C . The solid lines for low ATP concentrations (1.45–9.7 μM) are calculated assuming that the initial reaction follows monomolecular kinetics and the amounts of the initial burst of P_i liberation are equal to the horizontal bars given at the right ordinate.

ATP itself. Fig. 5 shows the temperature dependence of the initial reaction, when the molar ratio of ATP to myosin was one. The rate of initial hydrogen ion liberation was equal to that of initial P_i liberation, as our mechanism demanded. Furthermore, the activation energy for the formation of EP was rather high, namely about 24 kcal per mole, and the activation entropy was estimated to be +21.6 entropy units. The latter value suggests a conformational change in the formation of EP. The dependence of the rate of EP-

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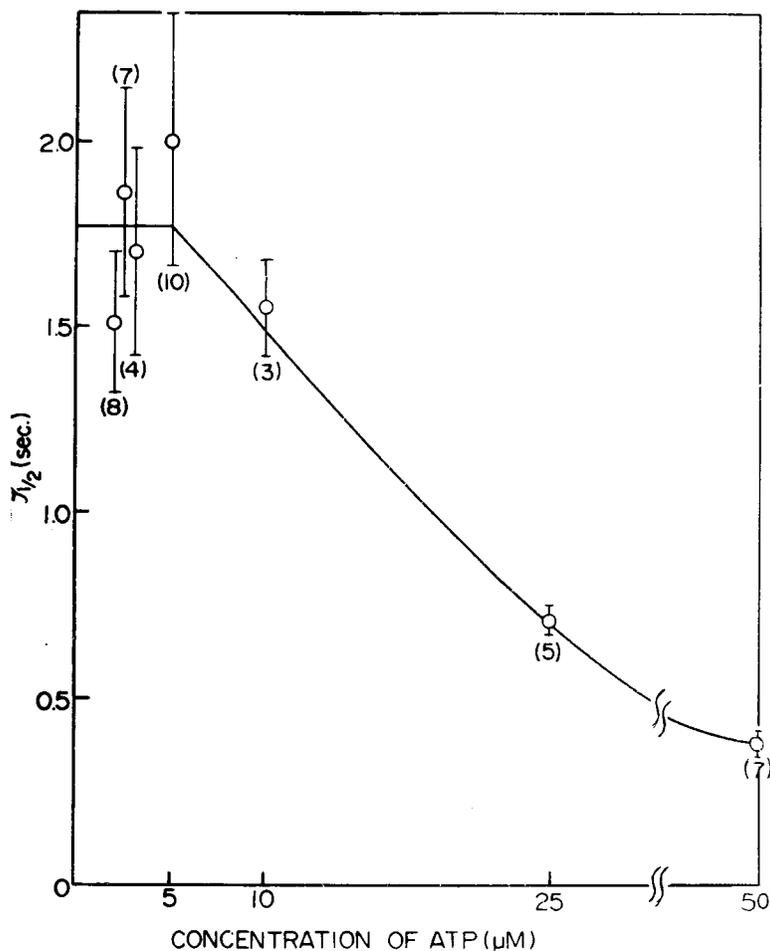


Fig. 4. Dependence of half-time, $\tau_{1/2}$, of initial rapid liberation of hydrogen ion on ATP concentration.

2.05 mg myosin/ml, 2 M KCl, 2 mM $MgCl_2$, 52 μ M cresol red, pH 8.3, 21°C. Open circles are mean values of several measurements (the number in parenthesis are the times of measurements) and flags indicate mean square errors.

formation on pH suggested the participation of one ionic group of pK 6.4 in EP-formation.

We studied the properties of EP by five different methods. One set of experiments was on the effect of *p*-nitrothiophenylation of myosin. If the initial phase of the reaction is the formation of a phosphoryl myosin, it might be possible to trap this labile phosphoryl intermediate with a nucleophilic reagent, and we used *p*-nitrothiophenol for this purpose. We found that

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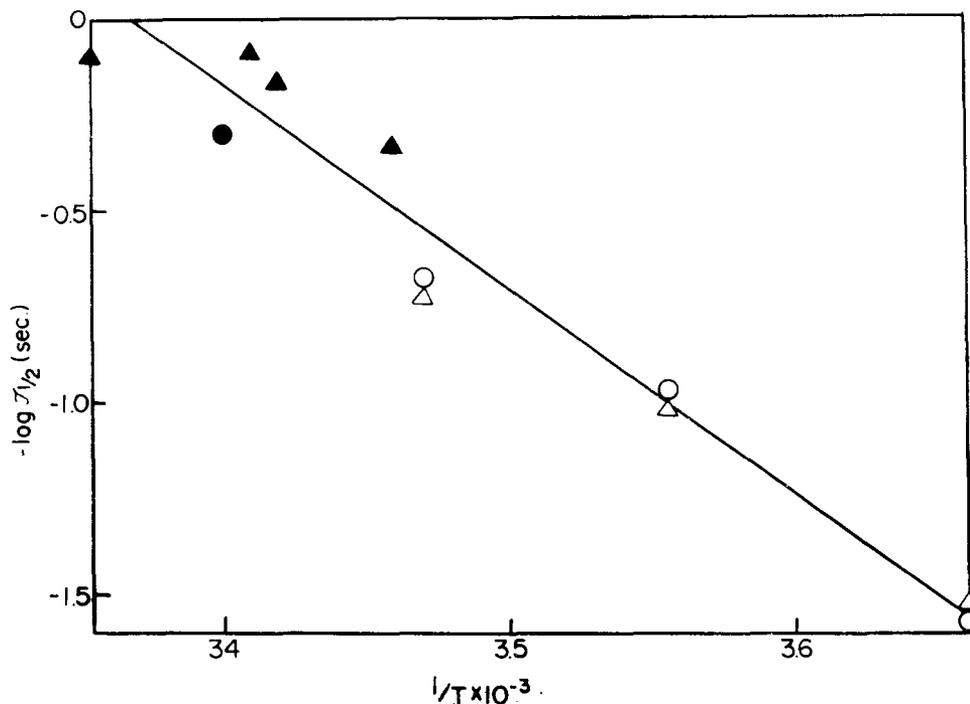


Fig. 5. Temperature-dependence of half-time, $\tau_{1/2}$, of P_1 and hydrogen ion liberation in the initial phase. 1.93–2 mg myosin/ml, 5 μ M ATP, 2 M KCl, 2 mM $MgCl_2$, pH 8.3. \circ , \triangle , $\tau_{1/2}$ of initial P_1 liberation in the presence of 20 mM Tris-HCl; \bullet , \blacktriangle , $\tau_{1/2}$ of initial H^+ liberation in the presence of 52 μ M cresol red. Circles and triangles are values measured with different preparations of myosin.

p-nitrothiophenol can combine with myosin only in the presence of Mg^{++} and ATP; that is, only under conditions where an initial rapid liberation of P_1 is seen.^{8,18} *p*-Nitrothiophenyl myosin with about one mole of *p*-nitrothiophenyl group per mole of myosin did not show an initial burst of P_1 liberation.^{19,20} On the other hand, the specific *p*-nitrothiophenylation did not affect properties of myosin-ATPase at the steady state, such as the maximum rate, and the pH-dependence.¹⁰ But the Michaelis constant was decreased by *p*-nitrothiophenylation. To clarify the binding site of *p*-nitrothiophenol in myosin, *p*-nitrothiophenyl peptides were obtained by hydrolysis with Nagarse and Pronase. We digested the peptides with amino peptidase and prolidase, and isolated the *p*-nitrothiophenyl amino acids. One mole of glutamic acid was found per mole of *p*-nitrothiophenol, and the amounts of other amino acids were much less than that of *p*-nitrothiophenol.²¹

These results, however, do not necessarily provide unequivocal evidence for the existence of a co-valently linked phosphoryl group in EP. For example, they can be explained, if we assume that EP is a myosin-metaphosphate complex, as suggested by BOYER, and that it is formed via a phosphoryl myosin which is attacked by *p*-nitrothiophenol. Therefore, in a second set of experiments we investigated the phosphate-exchange reaction between ATP and EP.²²⁾ Fig. 6 illustrates the procedures we adopted. In the first or control experiment, γ -P³²-labelled ATP with a total count, α , was added to myosin at a molar ratio of one to one, and the progress of P_i³² liberation was followed by stopping the reaction with trichloroacetic acid. In the second experiment, an extremely small amount of γ -P³²-labelled ATP, with a total count γ , almost equal to that of the control experiment, was added to myosin, at time t_0 after the addition of non-labelled ATP to myosin at a molar ratio of one to one, and the time-course of P_i³² liberation was determined. The quantity, Δ , was defined as the difference between the count of P_i³² at time t of the second

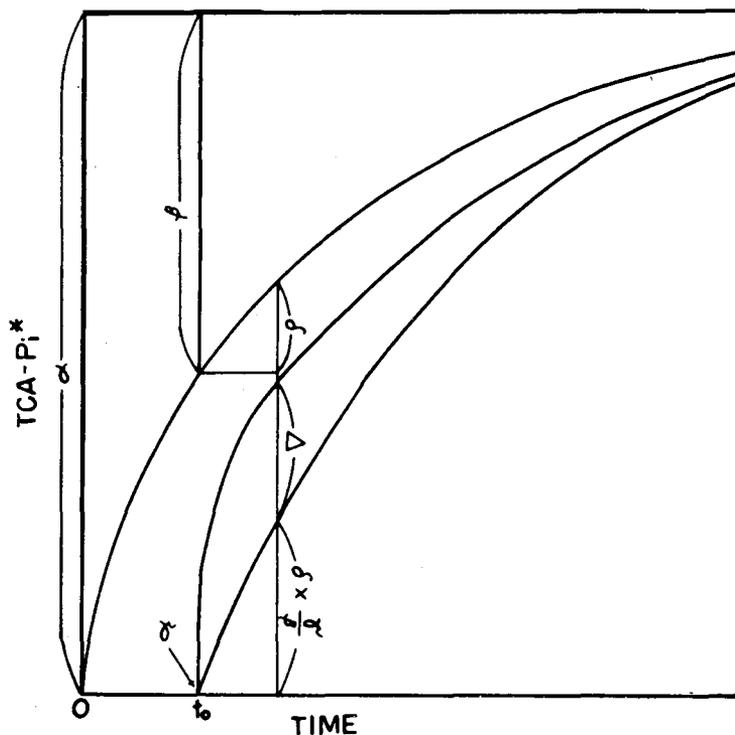


Fig. 6. Method of analysis of the results on the phosphate exchange reaction between ATP and EP.

For explanation see text.

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experiment and the calculated value, $\gamma \cdot \delta / \beta$, where β was the count of ATP^{32} remaining at time t_0 in the control experiment and δ was the count of P_i^{32} liberated during the period, $t - t_0$, in the control experiment. There are several lines of evidence indicating that the ES complexes are in equilibrium with myosin and ATP under our experimental conditions. Then, the difference, Δ , can be attributed to the exchange of phosphate between EP and P^{32} -labelled ATP. We found that the initial reaction of the myosin-ATP system was unaffected by preincubation for up to 3 hours. We also found that the time-course of P_i^{32} liberation in the second experiment was insensitive to a variation in the amount of radioactive ATP, of constant radioactivity, when the molar

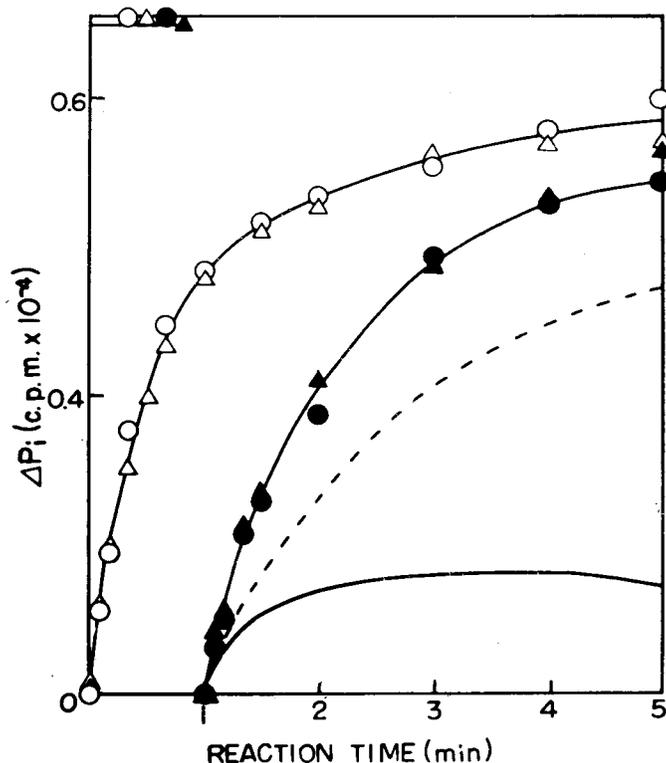


Fig. 7. Typical example of the examination of the phosphate-exchange reaction between EP and $\gamma\text{-P}^{32}\text{-ATP}$.

2 mg myosin/ml, 2.78 M KCl, 10 mM MgCl_2 , 20 mM Tris-HCl, pH 7.5, 0°C. ●, ▲, 0.22 μM of radioactive ATP was added 1 minute after the addition of 5 μM of non-radioactive ATP to myosin; ○, △, control experiment; ○, ●, 0.1 mM CaCl_2 ; △, ▲, 0.1 mM EGTA. -----, curve calculated as for Fig. 6; —, difference between the experimental values and the calculated ones. The radioactivity of ATP^{32} used in each experiment is indicated at the upper left of the figure.

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concentration of ATP was less than 5 per cent of that of myosin. Fig. 7 illustrates one example of determination of the difference, Δ . The contents of phosphate-exchangeable intermediate, $E\sim P$, in the total EP were calculated from the values of Δ . In the presence of high concentrations of KCl and $MgCl_2$, 15–20 per cent of the total EP was found to be able to exchange phosphate with ATP. The content of phosphate-exchangeable intermediate decreased with decreasing KCl concentration, but it was not affected by raising the temperature from 0° to 15°C, or by addition of a small amount of $CaCl_2$ or EGTA. Thus, we may conclude that the myosin-phosphate complex, EP, consists of two intermediates, a P-exchangeable myosin-phosphate complex and a P-nonexchangeable complex. Various lines of evidence we obtained, especially on the *p*-nitrothiophenylation of myosin mentioned previously, suggest that the former complex is formed by phosphorylation of the carboxyl group of one glutamic acid residue in the myosin molecule by ATP. The formation of the latter complex is not accompanied by over-all hydrogen ion liberation. Furthermore, the hydrolysis of simple acyl phosphates is generally assumed to proceed through the monomeric metaphosphate ion. Therefore, it seems possible that the P-nonexchangeable intermediate is a myosin-metaphosphate complex, as suggested by BOYER.

The life-time of EP was determined in a third set of experiments. From the mechanism which is shown in p. 326, it is expected that, in a system consisting of myosin and ATP in a molar ratio of one to one, the amount of P_i liberated should reach that of added ATP within a few seconds after the initiation of the reaction, because the phosphorylation step occurs very rapidly and is stoichiometric. Also the liberation of hydrogen ion should proceed at a much slower rate than the appearance of P_i , since the rate constant of hydrogen ion liberation, k_5 , should be smaller than the maximum velocity of steady state ATPase, that is, k_2+k_3 . Actually, the value of k_5 at 27°C, calculated from the time-course of hydrogen ion liberation, when ATP was added to myosin at a molar ratio of one to one, was one tenth of that of decomposition of E_1S by simple hydrolysis.¹⁹⁾ However, the possibility could not be excluded that the observed change in hydrogen ion concentration accompanied reactions other than EP-decomposition. Therefore, the life-time of EP was measured by another method with a myosin preparation which showed no phosphate-exchange reaction under the conditions used. We measured the time-course of P_i^{32} liberation, first, using a system in which one mole of radioactive ATP was added per mole of myosin, and second, using systems in which the addition of one mole of non-radioactive ATP per mole of myosin was followed, at varying intervals, by that of one mole of radioactive ATP.²²⁾

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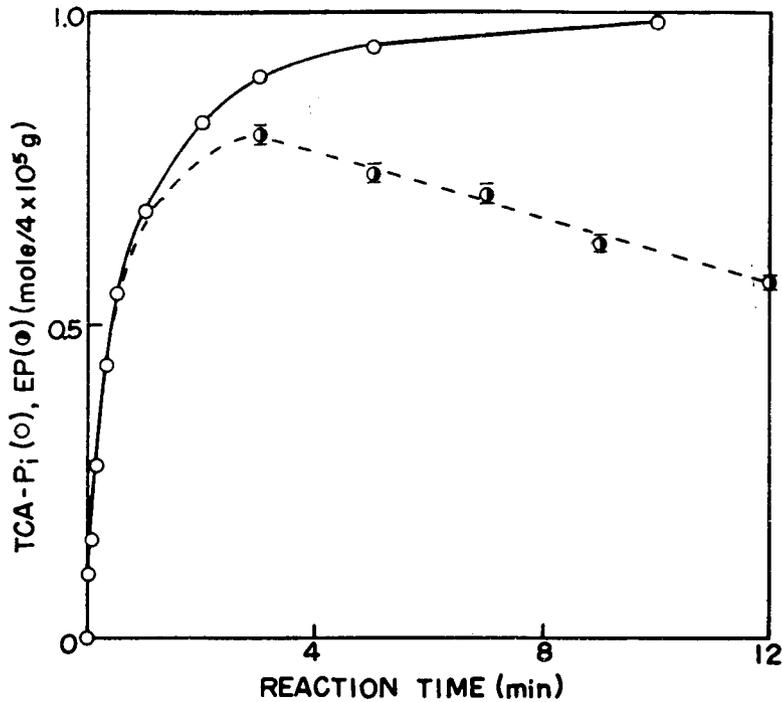


Fig. 8. Formation and decomposition of EP as a function of time.

2 mg myosin/ml, 1.08 M KCl, 5 mM MgCl₂, 20 mM Tris-HCl, pH 7.5, 0°C.
 O, Liberation of trichloroacetic acid-labile P_i³² after adding 5 μM γ-P³²-ATP to myosin; ●, amount of EP, calculated as described in the text.

In this case, the initial rate of P_i³² liberation was proportional to the amount of total EP, when corrections were made for the effect of ADP and for the rate of ATP-decomposition by simple hydrolysis. As shown in Fig. 8, the rate of EP-decomposition at 0°C was found by this method to be about one fifth that of simple hydrolysis of E₁S. These two results show that myosin is actually a double-headed enzyme.

The fourth experiment was to isolate EP. ATP was mixed with a myosin suspension in the presence of Mg⁺⁺ and a low concentration of KCl at a molar ratio of one to one. After incubating the mixture for 20 seconds, the ATP and ADP in the mixture were removed by adding a large amount of charcoal, and the protein precipitate was separated on a Millipore filter.¹⁹⁾ The precipitate on the filter was washed several times with buffer solution, and then trichloroacetic acid was poured onto it to decompose the phosphorylated intermediate. Then, the amount of P_i appearing in the filtrate was determined.

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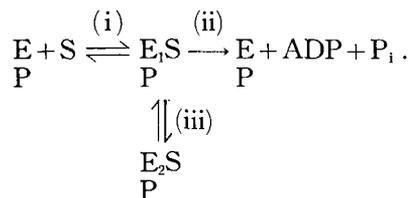
The content of trichloroacetic acid labile P_i and its decrease with time after stopping the reaction were consistent with the assumption that the trichloroacetic acid labile P_i was derived from EP.

Our fifth group of experiments were on the relation between the formation of EP and the myosin-catalyzed O^{18} exchange reaction discovered by Koshland and his co-workers.⁶⁾ Both the formation of EP and the O^{18} exchange reaction are inhibited by EDTA and PCMB. The intermediates of both these reactions are unstabilized by the binding of F-actin, but are unaffected by dinitrophenol. Furthermore, we have recently found that the dependencies of the stabilities of the EP of myosin and H-meromyosin on various divalent cations are quite similar to those of the exchange reaction obtained by KOSHLAND.⁶⁾ Therefore, it may be concluded that the O^{18} exchange reaction occurs at EP.

2. Interpretation

In the first part of this article, the reaction mechanism of the myosin-ATP system was analyzed. On the basis of this mechanism, we can now explain the complicated features of the myosin-ATPase reaction and its physiological function. The first problem to be treated is the mechanism of modification of myosin-ATPase.

Our studies on the pre-steady state of the myosin-ATP system indicated the existence of two types of myosin-ATP complex: E_1S and E_2S . Only the former seems to be a reaction intermediate of simple hydrolysis of ATP. Therefore, even at the steady state there are probably two myosin-ATP complexes, active and inactive in simple hydrolysis, since at the steady state EP contains no bound ADP:*)



A simple mechanism for modification of myosin-ATPase is the activation of step (ii) in the above scheme by a chemical modifier. The activation by monoiodoacetamide is of this type.²³⁾ The rate of the ATPase reaction at the

*) The binding of ATP with E seems to be somewhat different from that of ATP with EP, since the former is accompanied by adsorption of H^+ , while the latter is not accompanied by any change in H^+ concentration.

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steady state was markedly enhanced by the binding of 1 mole of monoiodoacetamide to 4×10^5 g of myosin, but the pattern of pH-activity curve was not significantly altered by the binding. These results indicate the activation of step (ii) by this reagent. However, the molecular mechanism for the activation of step (ii) remains to be clarified. One possible explanation for this is the following: for simple hydrolysis of ATP an interaction may be required between the triphosphate chain of ATP and an active group of myosin, such as the imidazole group of the histidine residue.^{24,25} If such an interaction is perturbed by a cysteine residue near the histidine, and if this perturbation is removed by blocking the cysteine residue with a chemical modifier, then the modifier should activate step (ii).

Modifiers of the second type shift the equilibrium of step (iii) from the E_2S side to the E_1S side. As is well known, myosin-ATPase in the steady state shows maximal activity at pH 6.0, and minimal activity at pH 7.5. Its activity is greatly enhanced by SH-reagents and EDTA, and the pH-activity curve of ATPase modified by these reagents lacks a depression in the pH-activity curve at neutrality (Fig. 9). We have explained these phenomena by assuming the existence of active and inactive myosin-ATP complexes in the steady state.²⁶⁻²⁸ The active complex can easily be formed at about pH 7.5, and its formation is inhibited by SH-reagents and EDTA. As already stated, SH-reagents and EDTA actually inhibited the formation of E_2SH^+ .

The third type of chemical modifier is the inhibitor of formation of EP.

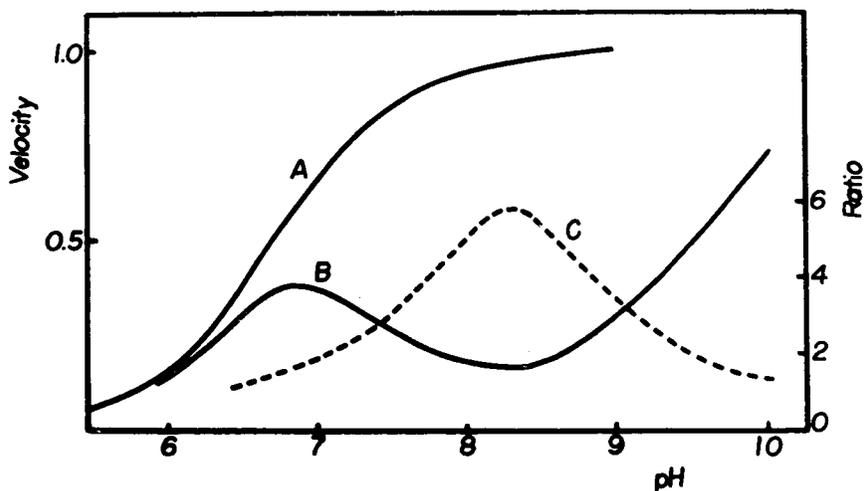


Fig. 9. The theoretical curves of pH-dependence of myosin-ATPase. A, in the presence of an activator; B, under control condition; C, ratio of A to B.

p-Nitrothiophenol is a modifier of this type. The fourth type is the accelerator of decomposition of EP. Trinitrobenzenesulfonate and F-actin belong to this type. These two types of modification will be described later in connection with the mechanism of the actomyosin-type of ATPase.

The second problem to be treated is the change in size and shape of actomyosin in a solution of high KCl concentration induced by the addition of ATP. The molecular mechanism of this change has been studied by many investigators. MORALES and we have presented several lines of evidence to show that the decrease in the intensity of light-scattering of natural actomyosin (myosin B) solution in 0.6 M KCl induced by ATP is mainly due to elongation of actomyosin particles.²⁹⁻³¹⁾ On the other hand, GERGELY and his co-workers have shown that at high ionic strength synthetic actomyosin dissociates into myosin and F-actin on the addition of ATP. As will be mentioned later, we can obtain a clue to the molecular mechanism of the reaction of the myosin-F-actin-ATP system by investigating the kinetic properties of the change in size and shape of actomyosin by ATP.

The kinetic study on the change of actomyosin solution induced by ATP was reported by us in 1952,^{32,33)} and later by MOMMAERTS. However, previous studies were made before the reaction mechanism of the myosin-ATP system had been established and good methods of rapid mixing of actomyosin with ATP had been developed. Quite recently, we reinvestigated the molecular mechanism of dissociation of synthetic actomyosin by ATP, using a new type of mixing chamber made to measure the change in the light-scattering intensity.³¹⁾

Fig. 10 shows the dependency of the amount and the rate of decrease of light-scattering on the concentration of ATP. Over wide variations in experimental conditions, the rate of dissociation of actomyosin induced by ATP was essentially the same as that of the initial burst of P_i and H^+ liberation on the addition of ATP, when corrections were made for the change in the concentration of free ATP by its binding with myosin. At high ionic strength, the decrease in the intensity of light-scattering increased with increase in the amount of added ATP up to one mole per 4×10^5 g of myosin (*cf.* 35). At higher ATP concentrations than this, the decrease in the intensity of light-scattering remained constant. When the concentration of myosin was varied with a fixed concentration of F-actin, the rate of decrease in the intensity of light-scattering seemed to be unaffected by changing the ratio of myosin to F-actin, but the amount of ATP required for the maximal decrease in the intensity of light-scattering increased in proportion to the amount of myosin. Therefore, we concluded that the phosphorylation of myosin by ATP is the

A Unified Theory for the Interaction Between Myosin and Adenosine Triphosphate

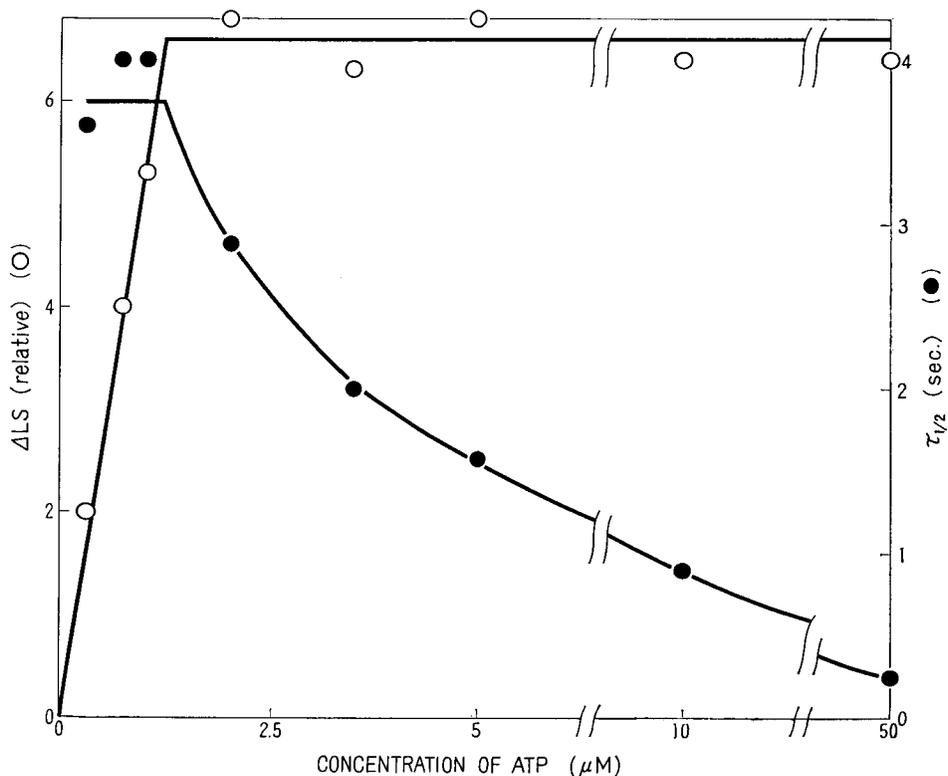


Fig. 10. Dependence of amount, Δ , and half-time, $\tau_{1/2}$, of decrease in light-scattering of actomyosin on ATP concentration.

0.4 mg myosin/ml, 0.2 mg F-actin/ml, 2 M KCl, 2 mM MgCl_2 , 25 mM Tris-HCl, pH 8.2, 22°C. O, Amount of decrease in light-scattering; ●, half-time of decrease in light-scattering.

rate-determining step in the dissociation of actomyosin induced by ATP, and that the rate of phosphorylation of myosin is scarcely affected by the presence of F-actin. The results that the rate of light-scattering drop in the presence of high concentration of EDTA, *i. e.* in the presence of extremely low concentration of true substrate ($\text{Mg}\cdot\text{ATP}$), was very slow and that its dependency on the concentration of ATP was of the second order supported this conclusion.³⁶⁾

We found that both the amount of ATP required for the maximal decrease in the intensity of light-scattering of actomyosin and the amount of the initial burst of P_i liberation in the actomyosin-ATP system increase with decrease in KCl concentration. Thus, at high ionic strength all the complexes

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chain are necessary for contraction of myofibrils.^{12,40-42)}

To confirm the importance of phosphorylation of myosin in muscle contraction, we recently made three more experiments. The first experiment was on the effect of F-actin on phosphoryl myosin. From WEBER's work it is now generally accepted that the actomyosin-type ATPase is directly coupled with muscle contraction. We also demonstrated recently that the amount of shortening of isolated myofibrillar fragments is proportional to the amount of ATP splitting by the actomyosin-type ATPase.⁴³⁻⁴⁵⁾ We have already given kinetic evidence that in the actomyosin-type ATPase reaction ATP is mainly hydrolyzed through the route involving phosphoryl myosin.^{8,39)} Actually, we recently showed that the rate of the decomposition of phosphoryl myosin is greatly increased when F-actin binds to myosin.¹⁹⁾ The time-courses of hydrogen ion and P_i liberation after adding ATP to actomyosin in the presence of Mg^{++} and a low concentration of KCl were measured, and it was found that unlike in the myosin-ATP system, almost all the hydrogen ion liberation occurred within 10 seconds. This means that F-actin greatly accelerates step 5 and that the rate constant of the decomposition of EP of actomyosin, that is k_5 , becomes more than 100 times higher than that of myosin.

Our second set of experiments was on the effect of *p*-nitrothiophenylation of myosin on a model contractile system.²⁰⁾ As mentioned previously, *p*-nitrothiophenyl myosin contains about one mole of *p*-nitrothiophenyl group per mole of myosin and shows no phosphorylation, though in the steady state its ATPase is equal to that of control myosin. Furthermore, we found by ultracentrifugal analysis that *p*-nitrothiophenyl myosin binds with F-actin, just as the control myosin does. However, even at low ionic strength and in the presence of Mg^{++} , actomyosin which was reconstituted from *p*-nitrothiophenyl myosin and F-actin showed only myosin-type ATPase activity and its activity was only 4 per cent of that of the control actomyosin. But in the presence of EDTA, which prevents the phosphorylation, both *p*-nitrothiophenyl actomyosin and the control actomyosin showed myosin-type ATPase activity and their activities were the same. The superprecipitation of actomyosin was followed by the turbidity method, and it was found that the superprecipitation was completely prevented by *p*-nitrothiophenylation.

In a third set of experiments, we compared the effects of pH and temperature on EP formation with those on the actomyosin-type and the myosin-type of ATPase.³⁾ As mentioned previously, the pH-dependence of EP formation suggested participation of a functional group with a pK of 6.4, and the activation entropy of the step was estimated to be +21.6 e.u. On the other hand, the pH-activity curve of myosin-ATPase at the steady state is well known to

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have a maximum value at pH 6.0 and a minimum value at pH 7.5, and we recently estimated the activation entropy to be -24.6 e.u.³⁾ But, the rate of the actomyosin-type ATPase reaction increased with increasing pH and the half maximum value was obtained at pH 6.1, and the activation entropy was found to be $+45.5$ e.u. These results suggest that the step of formation of EP is rate-determining in the actomyosin-type of ATPase.

Recently, A. G. SZENT-GYÖRGYI has emphasized the role of the ADP of F-actin in superprecipitation of actomyosin. However, our experiments have shown that the ADP which is bound to F-actin does not participate directly in superprecipitation of actomyosin or in the actomyosin-type of ATPase reaction. ADP free F-actin prepared by prolonged dialysis could activate the deoxy-ATPase activity of myosin just as control F-actin did.⁴⁶⁾ As shown in Fig. 11, actomyosin reconstituted from myosin and ADP free F-actin superprecipitated on the addition of deoxy-ATP just as normal actomyosin did.

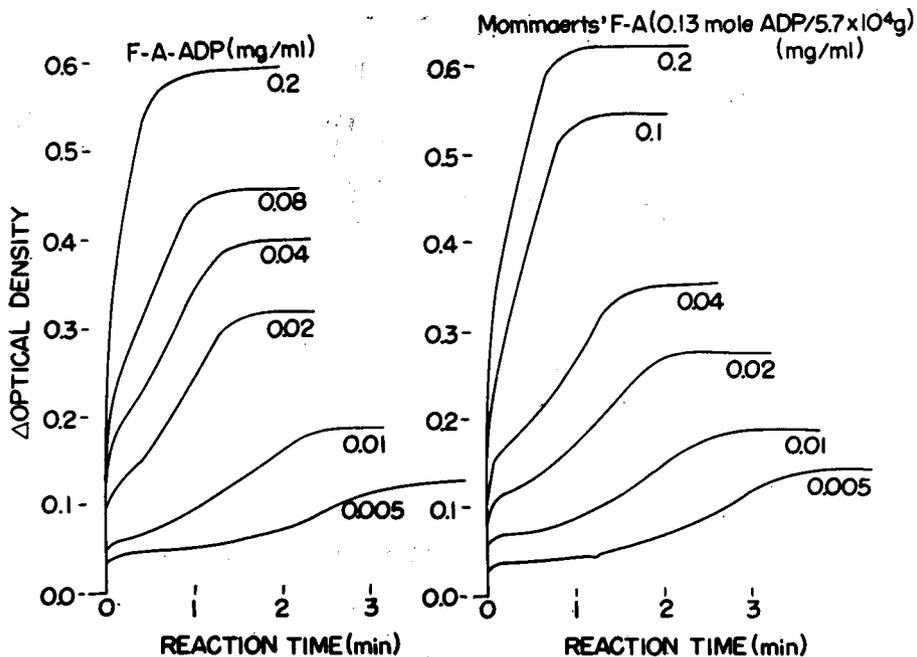


Fig. 11. Time-course of deoxy-ATP induced superprecipitation of actomyosin reconstituted from myosin and MOMMAERTS' F-actin free from ADP.

0.8 mg myosin/ml, 0.1 mM deoxy-ATP, 0.1 M KCl, 1 mM MgCl₂, 0.11 mM CaCl₂, 0.1 mM EGTA, 5 mM Tris-maleate, pH 7.0, 25°C. Concentrations of F-actin are indicated in the figure.

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But the incorporation of deoxy nucleotide into actin did not occur. BÁRÁNY also found that actomyosin made with myosin and ADP free F-actin superprecipitated on addition of CTP. He found a typical actomyosin-type of CTPase, though neither CTP nor CDP combined with actin. Under conditions in which the superprecipitation of actomyosin took place, no formation of C^{14} -ITP or C^{14} -ATP was detected in the F-actin- C^{14} -IDP-myosin-ATP system or the F-actin- C^{14} -ADP-myosin-ATP system.⁴⁷⁾ These two experiments clearly show that the ADP of F-actin does not participate in the biochemical function of actin.

Furthermore, our recent experiments have indicated that the remarkable activation of myosin-ATPase by trinitrophenylation in the presence of Mg^{++} is due to acceleration of decomposition of EP by trinitrophenylation.⁴⁸⁾ As was mentioned before, trinitrophenylation of myosin occurred at one specific lysine residue in the myosin molecule. We have found that the steady state rate of the reaction of ATPase of myosin without trinitrophenylation is not dependent on the size of the initial burst. However, the rate of Mg^{++} -ATPase of trinitrophenyl myosin decreased with decrease in the size of the initial burst on *p*-nitrothiophenylation or prolonged incubation of myosin, and it reached the control level of untreated myosin, when the size of the initial burst became zero. Thus, the effect of F-actin on ATPase can be substituted by a chemical modification of myosin. We have already shown that the trinitrophenylation of myosin does not affect the binding of myosin to F-actin.¹³⁾ Furthermore, PERRY has shown that the active site of ATPase of myosin is different from the site for the binding with F-actin. Therefore, F-actin is an allosteric effector of the myosin-ATP system.

We must now discuss the molecular mechanism of superprecipitation of actomyosin induced by ATP. It has generally been accepted to be a phenomenon corresponding to the contraction in a disorganized system. However, we have shown that, when KASAI's F-actin free from ADP was used, the deoxy-ATP induced superprecipitation of actomyosin was scarcely affected by removal of ADP from F-actin.⁴⁶⁾ But the stimulation of the deoxy-ATPase activity of myosin by F-actin decreased with decrease in the ADP content of F-actin by KASAI's method. Actually, using KASAI's F-actin completely free from ADP, we found that only 0.1 mole of deoxy-ATP per mole of myosin was hydrolyzed by the actomyosin-type of deoxy-ATPase before completion of superprecipitation of actomyosin. Actin which was polymerized at extremely low protein concentrations behaved like KASAI's F-actin free from ADP, although it contained bound ADP.⁴⁷⁾ Thus, the superprecipitation of actomyosin can occur without the participation of the actomyosin-type of enzymic

activity. But the formation of EP is an obligatory step for superprecipitation, as mentioned before. We also found that cardiac myosin B often shows a clearing response to ATP, while it shows the actomyosin type of ATPase.⁵⁾

Our following experiments also indicated the difference between the superprecipitation of actomyosin and the contraction of skeletal muscle.⁴⁹⁾ The rate of superprecipitation decreased as the concentration of myosin B decreased, and reached zero at below the critical protein concentration. The superprecipitation of myosin B was strikingly accelerated by the addition of a minute amount of superprecipitated myosin B. As shown in Fig. 12, the accelerating effect of a minute amount of superprecipitated myosin B was increased still more by sonication and even the addition of $0.3 \mu\text{g}/\text{m}\ell$ of superprecipitated myosin B increased the rate of superprecipitation of $0.3 \text{ mg myosin B}/\text{m}\ell$ by 20–30 fold. The acceleration disappeared completely when the superprecipitated myosin B used as an accelerator was dissolved in 0.6 M KCl . The minute amount of superprecipitated and sonicated myosin B used as an accelerator

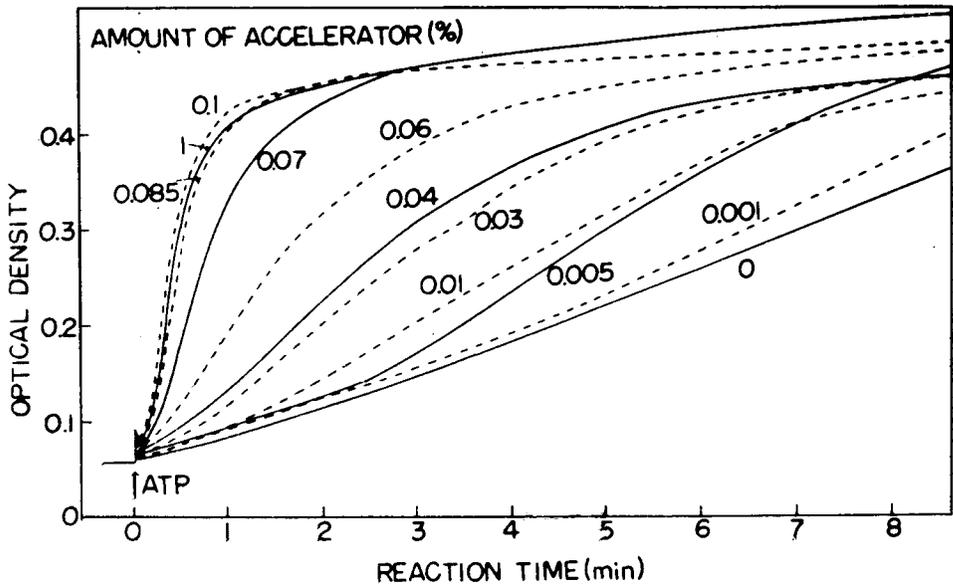


Fig. 12. Acceleration of superprecipitation of myosin B induced by a small amount of myosin B previously superprecipitated and sonicated.

$0.3 \text{ mg myosin B}/\text{m}\ell$, 0.5 mM ATP , 0.06 M KCl , 2 mM MgCl_2 , $30 \mu\text{M EGTA}$, $20 \text{ mM Tris-maleate}$, $\text{pH } 7.0$, 25°C . Myosin B which has been superprecipitated and sonicated for 15 minutes was added as an accelerator at the concentration indicated in the figure.

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did not affect the ATPase activity. In the presence of a minute amount of superprecipitated myosin B added as an accelerator, the superprecipitation followed first order kinetics, and the rate became independent of the myosin B concentration. From these results, we can conclude that the molecular mechanism of superprecipitation of myosin B is different from that of contraction of skeletal muscle, and that the superprecipitation in a nucleated-growth process.

The final problem to be interpreted on the basis of our reaction mechanism is the molecular mechanism of muscle relaxation. In connection with this, at first some properties of a new F-actin will be mentioned. We recently isolated the new F-actin from the "minor component" of metin of SZENT-GYÖRGYI and KAMINER and AZUMA and WATANABE, by repeated ultracentrifugal separation without G-F transformation.⁵⁰⁾ The unique properties of this F-actin are destroyed by dialysis against ATP solution which is usually used for G-F transformation. This F-actin appeared as a typical double-stranded helical polymer, it changed the aggregation-state of myosin at low ionic strength, and its effect was very greatly modified by the further addition of ATP. The round aggregate of myosin obtained by the addition of F-actin changed instantaneously to a beautiful net-work of filaments on the addition of ATP. We found that the amount of F-actin necessary to produce this change was 1-2 per cent of the myosin present. The change produced by the addition of ATP was reversible and it required Mg^{++} and a trace of Ca^{++} (about $1 \mu M$), though superprecipitation of normal actomyosin is irreversible. Furthermore, the maximum activation of myosin-ATPase by this F-actin was observed at a molar ratio of myosin to actin monomer of 1:1, while the activation by normal F-actin is observed at a ratio of 1:2.^{36, 51)}

A. WEBER and EBASHI have shown that under physiological conditions the superprecipitation of actomyosin induced by ATP and the activation of myosin-ATPase by F-actin require a small amount of Ca^{++} . Later, EBASHI and others showed the necessity of new protein factors for these two phenomena. However, the reconstitution of the system from new protein factors and actomyosin, from which the factors were removed in advance, has usually been incomplete. The ATPase activity of the complex of our new F-actin and myosin was completely dependent on Ca^{++} , and Ca^{++} was necessary for acceleration of the decomposition of EP. When the F-actin was treated with a minute amount of trypsin for a short time, the activating effect of the actin on myosin-ATPase disappeared almost completely both in the presence and the absence of Ca^{++} (Fig. 13). After the trypsin-treatment the F-actin was incubated with the minor component of metin, and the actin was isolated

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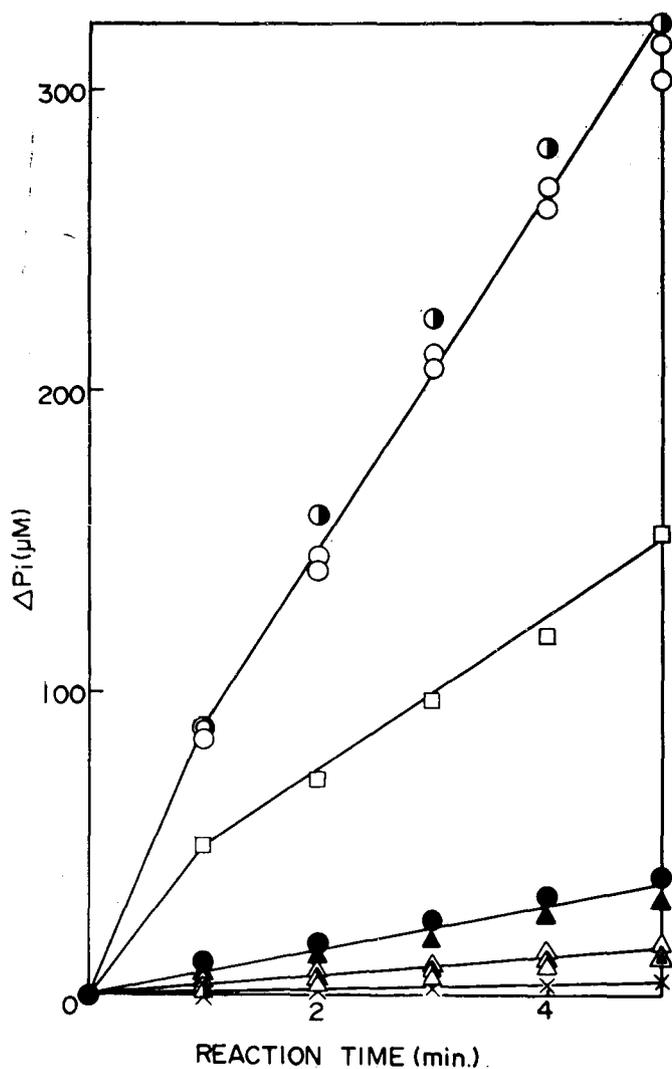


Fig. 13. Activation by Ca^{2+} of ATPase of the myosin-new F-actin system.

0.2 mg myosin/ml, 0.04 mg new F-actin/ml, 0.5 mM ATP, 0.05 M KCl, 2 mM MgCl_2 , 20 mM Tris-maleate, pH 7.0, 25°C. ○, □, △, new F-actin; ●, ▲, new F-actin treated with trypsin; ●, ▲, new F-actin treated with trypsin was incubated with metin; ×, no F-actin was added. ○, ●, ●, 0.1 mM CaCl_2 ; △, ▲, ▲, 0.1 mM EGTA; □, ×, no divalent cation was added.

again by ultracentrifugation, since metin contains "EGTA-sensitizing" protein factors. Then, the original activating properties of actin on ATPase were

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completely recovered.⁵²⁾ These results suggest the usefulness of this new type of F-actin in investigation for characterization of "EGTA-sensitizing" protein factors.

We must briefly discuss the reaction mechanism of relaxing factors on the basis of our reaction scheme. Our reaction mechanism suggests that muscle contraction can be prevented either by inhibition of formation of phosphoryl myosin or by inhibition of acceleration of decomposition of phosphoryl myosin induced by F-actin. It was mentioned previously that the formation of phosphoryl myosin was scarcely affected by the binding of myosin to F-actin. Furthermore, we have found that the phosphorylation of myosin by ATP was unaffected even by the presence of both F-actin and the EGTA-sensitizing factors. We found that in the presence of the EGTA-sensitizing factors the substrate inhibition of actomyosin-type of ATPase becomes to be markedly dependent on free Ca^{++} , and that in the absence of Ca^{++} the activity is severely inhibited by the presence of high concentration of the substrate itself. Thus, in the presence of the EGTA-sensitizing factors and high concentration of ATP, the actomyosin type of ATPase, *i. e.* the decomposition of phosphoryl myosin, is severely inhibited by the removal of free Ca^{++} . This induces the dissociation of myosin from F-actin, *i. e.* relaxation of muscle models, as easily understood from our reaction mechanism given in p. 340. Therefore, it seems reasonable to conclude that under physiological conditions, muscle contraction is regulated by regulation of the rate of decomposition of phosphoryl myosin, *i. e.* by modifying the interaction between myosin and F-actin.

3. Molecular Mechanism of Muscle Contraction

In the preceding part, the phosphorylation of myosin by ATP was shown to be the primary biochemical reaction of muscle contraction. As pointed out by MORALES, it seems impossible to explain such a rapid and large displacement of the relative positions of myosin and actin filaments in contracted myofibrils as observed of HANSON and HUXLEY, only by formation of covalent linkages in contractile proteins. Furthermore, as discussed in our previous review,³⁹⁾ the F-actin filament seems to remain unchanged during contraction. Recent X-ray analysis of structure of muscle fiber during the active state made by HUXLEY and ELLIOTT supported this view. Therefore, to develop a molecular mechanism of muscle contraction, the following problem must be elucidated: how the secondary and tertiary structures of myosin are changed by its reaction with ATP and F-actin.

We first studied the conformational changes induced by ATP or F-actin

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in the myosin molecule by measuring the optical rotatory dispersion. We found that the rotatory power of myosin changes several per cent on the addition of F-actin or ATP.^{53,54)} However, the magnitude of change was rather low and varied with the preparation of myosin.⁵⁵⁾ Recently, MORALES and his associates have obtained evidence for a conformational change in myosin induced by substrates and modifiers, using the method of spin-labelling of myosin.

Before discussing the conformational change in myosin molecules bound to F-actin filaments, our recent work on the binding of H-meromyosin to F-actin at low ionic strength must be mentioned.⁵¹⁾ We measured the intensity of light-scattering and the ATPase activity of the F-actin-H-meromyosin-ATP system. We found that at low ionic strength acto-H-meromyosin dissociates only partially on the addition of ATP, especially when PCMB- β -mercaptoethanol treated H-meromyosin is used. Six years ago, we reported that on treatment of myosin with PCMB and β -mercaptoethanol the intrinsic Ca^{++} is removed from myosin and the reaction of the myosin-F-actin-ATP system becomes remarkably insensitive to free Ca^{++} and ionic strength.^{56,57)} We measured the weight-average molecular weight of acto-H-meromyosin and concluded that at low ionic strength the binding of H-meromyosin to F-actin is co-operative,

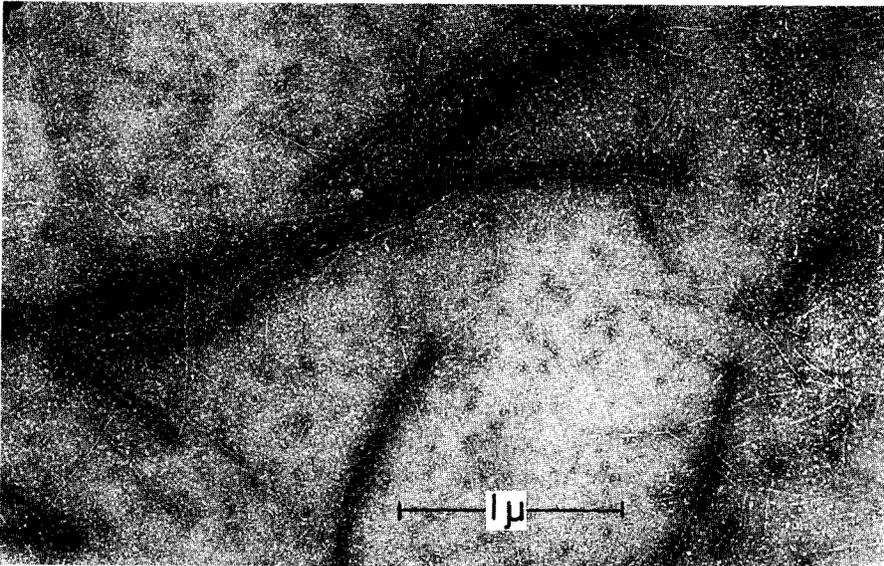


Fig. 14. Electron-microscopic photograph of acto-H-meromyosin.
0.05 mg H-meromyosin/ml, 0.15 mg F-actin/ml, 5 mM KCl, 1 mM MgCl_2 ,
10 mM Tris-HCl, pH 7.5.

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that is, there are only two kinds of F-actin filament. In one, the binding sites are completely occupied by H-meromyosin, and in the other, the binding sites are completely empty, when less than the saturation amount of H-meromyosin is added. This was supported by electron microscopic studies on acto-H-meromyosin, as shown in Fig. 14.⁵⁸⁾ This co-operative nature of binding seems to be important in elucidating the molecular mechanism of the actin-myosin-ATP system.

Basing on these results, we have made some electron-microscopic observations on the F-actin-H-meromyosin-ATP system.⁵⁸⁾ In the absence of ATP, acto-H-meromyosin showed an arrow-head structure. On the addition of ATP, the complex of F-actin with PCMB- β -mercaptoethanol treated H-meromyosin partially dissociated. However, in the presence of ATP the projections of H-meromyosins bound to F-actin were apparently perpendicular to the axis of the F-actin filament, and the shape of H-meromyosin was rather spherical. We could not exclude the possibility that these remarkable changes in H-meromyosin linked with F-actin on the addition of ATP are artifacts due to the negative staining technique, accompanied with partial dissociation of acto-H-meromyosin. But, the state of H-meromyosin, which is linked to F-actin in the absence of ATP and shows the arrow-head type structure, will be named the β state, and that of H-meromyosin which is phosphorylated and linked perpendicularly to F-actin with the spherical shape will be named the α state.

As described previously, ATP phosphorylates the carboxyl group of a specific Glu of myosin, and F-actin greatly accelerates the hydrolysis of phosphoryl myosin. Furthermore, the accelerating effect of F-actin has been shown to be due to the effect of F-actin on the secondary structure of myosin. Then, the actomyosin-type of ATPase reaction, that is, the decomposition of EP, which is activated by the allosteric effect of F-actin, may be coupled with the movement of protein filaments. This was tested in an experiment on the dependency of myofibrillar ATPase activity on the sarcomere length.⁵⁹⁾ Under our experimental conditions, the diffusion of ATP into the fiber was shown not to be the rate-determining step of ATPase reaction. As shown in Fig. 15, the ATPase activity of glycerinated fiber bundles decreased with increase in sarcomere length of more than 2.5μ . This decrease is attributable to a decrease in the length of overlap of F-actin and myosin filaments. When the sarcomere length was less than 2μ , the ATPase activity decreased as the sarcomere length decreased. HUXLEY reported that the muscle fiber does not develop tension when the sarcomere length reaches 1.3μ . Even in this state the binding of myosin to F-actin may not be disrupted. Therefore, it was

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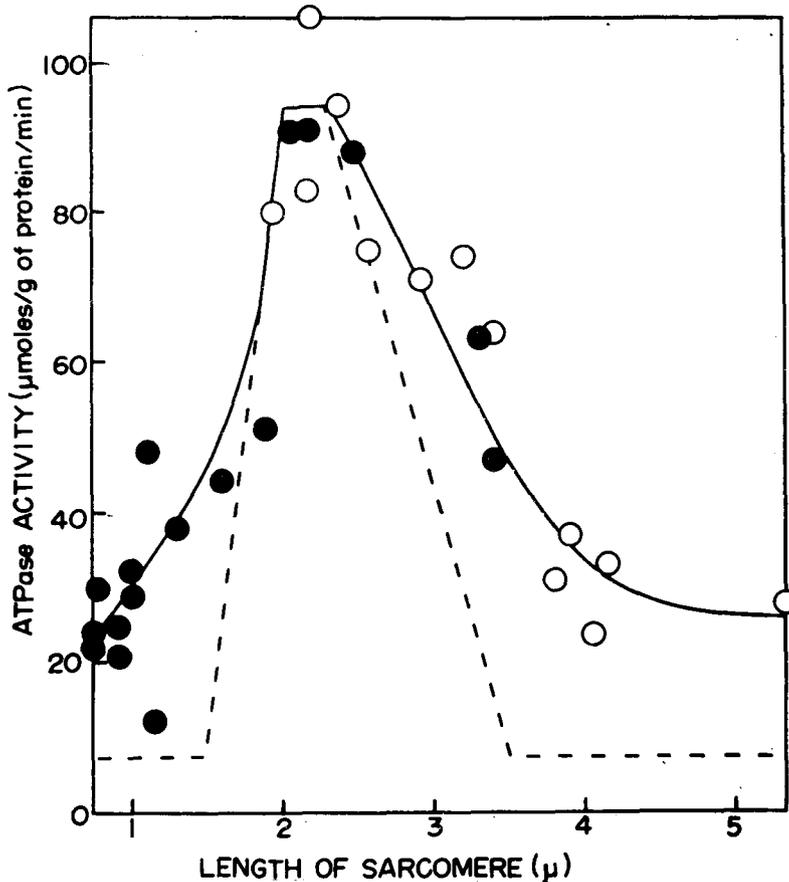


Fig. 15. Dependence of myofibrillar ATPase activity of glycerol-treated fiber bundles on sarcomere length.

0.6–2.35 mM ATP, 1.0 mM phosphoenol pyruvate, 8.4–73 μ g pyruvate kinase/ml, 50 mM KCl, 2.0–3.35 mM $MgCl_2$, 20 mM Tris-maleate, pH 7.0, 25°C. The broken line is the theoretical one deduced from electron microscopic observations by HUXLEY *et al.*

concluded that both ATPase activity and development of tension are coupled with sliding of actin filaments past myosin filaments.

Thus, the facts and suggestions on which our molecular mechanism of muscle contraction rests, are as follows: (i) Muscle contraction is coupled with the phosphorylation of myosin by ATP and its dephosphorylation induced by an allosteric effect of F-actin. (ii) H-Meromyosin in the β state forms a complex with F-actin of an arrow-head structure, and the molecular shape of H-meromyosin is slender. H-Meromyosin, which is phosphorylated and

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linked to F-actin, is in the α state. In this state, the projection of H-meromyosin from the F-actin are perpendicular to the axis of F-actin, and the shape of H-meromyosin is spherical. (iii) The movement of the head part of the myosin molecule during muscle contraction has been suggested by ELLIOTT and HUXLEY from their X-ray analysis of living muscle in the active state. The movement of the head of the myosin molecule during contraction was also suggested by us from the dependency of myofibrillar ATPase activity on sarcomere length, as was mentioned previously. (iv) The linkage between F-actin and myosin is easily broken by the phosphorylation of myosin by ATP. However, the strength of the linkage depends greatly on the conformation of myosin. The dependency on Ca^{++} of the binding of myosin with the complex of F-actin and EGTA-sensitizing factors in the presence of high concentration of ATP has been demonstrated by many workers, especially by EBASHI and A. WEBER. (v) To operate the cyclic process of contraction and relaxation, the linkage between myosin and F-actin must be broken after the energy-coupled transconformation of the head part of the myosin molecule, and must be reformed, when the conformation of the head part of the myosin molecule returns to the original state.

A molecular mechanism of muscle contraction by the operation of a cycle, in which the transconformation of the myosin molecule takes place on its interaction with ATP and F-actin, was first proposed by us^{39,60,61} and then by DAVIES. Our previous molecular model of muscle contraction deduced from

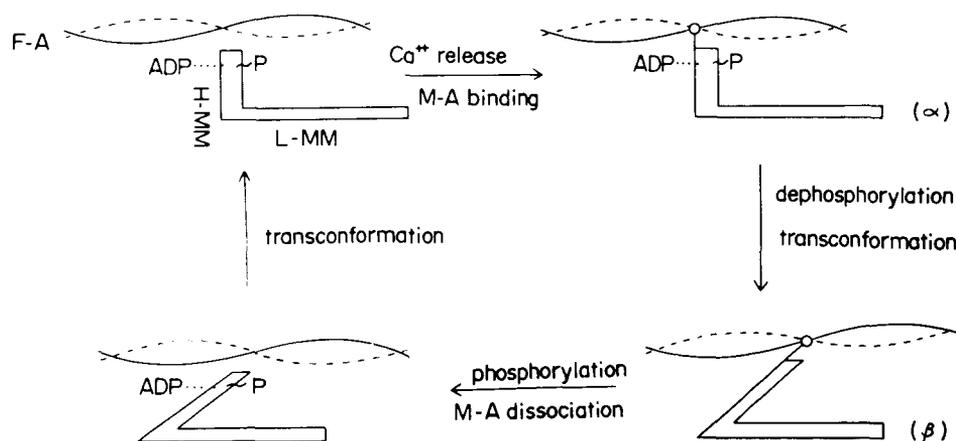


Fig. 16. A molecular mechanism of muscle contraction.

F-A (or A) and M are F-actin and myosin. H-MM and L-MM are H-meromyosin and L-meromyosin, respectively. The thick filament is formed by the binding of L-MM to each other, and the thin filament is formed of F-A and "EGTA-sensitizing factors".

the above general idea is modified on the basis of new information described in this article and is illustrated in Fig. 16. The system works as follows: (i) In the resting state, myosin is phosphorylated and its conformation is in the α state. (ii) When Ca^{++} is set free in the contractile system by excitation, a linkage between myosin and actin is formed. (iii) The phosphoryl myosin is rapidly dephosphorylated by the allosteric effect of F-actin, and this energy-releasing process accompanies the transconformation of myosin from the α state to the β state. Actin filaments are interdigitated into myosin filaments by this transconformation of myosin molecules, because of the polarity of the orientation of molecules in myosin as well as in actin filaments. (iv) Myosin is rephosphorylated by ATP. Even in the presence of Ca^{++} the linkage of phosphoryl myosin in the β state with F-actin is very weak, and the linkage is broken. (v) The transconformation of phosphoryl myosin from the β state to the α state occurs automatically, and the whole system returns to the original state. The cycle is repeated.

It should be added that, according to our recent kinetic work, the Na^+ - K^+ dependent ATPase of the membrane is also a double-headed enzyme.⁶²⁾ It hydrolyzes ATP by simple hydrolysis in the presence of Na^+ and K^+ at relatively low temperatures or in the presence of Na^+ alone over the entire temperature range. However, it hydrolyzes ATP through the phosphorylated enzyme in the presence of Na^+ and K^+ at high temperatures. Furthermore, we have recently demonstrated that rather a large number of carboxyl groups of Glu and Asp of this enzyme preparation are activated by $\text{Mg}\cdot\text{ATP}$, and the activation is dependent on K^+ and Na^+ .⁶³⁾ Our kinetic work has also shown that the reaction scheme of Ca^{++} -dependent ATPase (E) of sarcoplasmic reticulum is: $\text{E} + \text{S} \rightleftharpoons \text{ES}$, $\text{ES} + \text{Ca} \rightleftharpoons \text{ESCa} \rightleftharpoons \text{E}\sim\text{P} + \text{Ca} + \text{ADP} \rightarrow \text{E} + \text{P}_i + \text{Ca} + \text{ADP}$, where S is $\text{Mg}\cdot\text{ATP}$ complex.⁶⁴⁾ We have recently isolated $\text{E}\sim\text{P}$ as a reaction intermediate and have shown that $\text{E}\sim\text{P}$ is an acyl-phosphate compound. In the presence of Ca^{++} , this enzyme showed a large amount of initial burst of ATP-splitting, while the formation of $\text{E}\sim\text{P}$ showed a lag phase.⁶⁵⁾ The molecular mechanism of active transport of cation by these ATPases is not yet clear. But these results strongly suggest the importance of phosphorylation and activation of a large number of carboxyl groups of the protein by ATP in the active transport. About 30 years ago, HOPKINS emphasized the importance of molecular dynamics in considering the function of protein. In our previous review,^{59,60)} we have proposed to call a protein a "transconformer", when its physiological function is due to a conformational change, and suggested that all of the energy transformation in biological systems can be attributed to the function of protein as a "transconformer", accompanied by

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energy-liberation resulting from ATP-hydrolysis. The general principles and mechanisms of energy transformations in physiological functions, which are coupled with ATP-splitting, will be discussed in detail in a separate review in the near future.

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