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# ON THE ACTIVE SITE OF MYOSIN A ATPase<sup>\*)</sup>

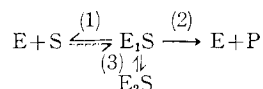
By

Naomi AZUMA and Yuji TONOMURA<sup>\*\*)</sup>

(Received Oct. 12, 1962)

## Abstract

The experimental results on kinetic properties of myosin A ATPase were reviewed and analyzed according to the following reaction scheme:



where E, S and P are the active site of the enzyme, the substrate and the product, respectively. Various complicated properties of myosin A ATPase were easily explainable by this mechanism. The effects of structure of the substrate on the reaction steps (2) and (3) were analyzed using several synthetic ATP analogs as the substrate. Furthermore, the relation of secondary structure and the enzymic activity was inferred from the changes in enzymic properties by specifically and locally increasing or decreasing the secondary structure around the active site on adding the modifiers of the enzymic action or changing the medium.

ENGELHARDT and LJUBIMOVA<sup>1)</sup> have demonstrated that the structural protein of muscle, myosin, catalyzes the hydrolysis of ATP into ADP and P<sub>i</sub>. Since then, many investigations<sup>2,3)</sup> have been made on this enzymic action and there can be little doubt that myosin plays the most fundamental part in muscular contraction. In order to clarify the mechanism of muscular contraction at the molecular level, it is urgently needed to inquire properties of myosin A ATPase, especially its kinetic behaviors and the primary and secondary structure of the peptide chain around the active center. We already suggested<sup>4)</sup> that myosin A is a double headed enzyme and the hydrolysis of ATP by myosin A passes through two routes; the one is hydrolysis through the phosphoryl

\*) In this paper the following abbreviations are used: ATP, adenosine triphosphate; ATPase, adenosinetriphosphatase; ADP, adenosine diphosphate; ITP, inosine triphosphate; GTP, guanosine triphosphate; CTP, cytidine triphosphate; UTP, uridine triphosphate; NTP, nucleoside triphosphate; RTP, ribose triphosphate; dimethyl ATP, 6-dimethylamino-9-β-D-ribofuranosyl purine 5'-triphosphate; BTP, 9-(4'-hydroxybutyl)-6-amino purine 4'-triphosphate; TP, inorganic triphosphate; EDTA, ethylenediamine tetraacetic acid; PCMB, p-chloromercuribenzoate; TBS, 2,4,6-trinitrobenzene sulfonate; TNP, trinitrophenol.

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myosin A and the other direct hydrolysis without going through the phosphoryl myosin A. We have proposed a molecular mechanism of muscle contraction based on the hydrolysis of ATP via the phosphoryl protein<sup>9</sup>. In this paper we shall focus our attention principally on the mechanism of hydrolysis of ATP without the phosphoryl intermediate of myosin A.

### **1. Number of ATP-Binding Site**

The molecular weight, radius of gyration, sedimentation constant and intrinsic viscosity of our myosin A extracted and purified from rabbit striated muscle were  $5.9 \times 10^5$ , 1,058 Å, 6.11 S and 2.30 (100 ml/g), respectively<sup>6</sup>. In order to know how ATP combines with myosin A, it is necessary to determine how many moles of ATP can bind per one mole of myosin A. The method of equilibrium dialysis has been proved to be the most valuable one to measure the number of binding site of enzyme. In this method, however, ATP cannot be used as it is hydrolyzed by myosin A. Pyrophosphate was used as the substrate instead of ATP since it is not hydrolyzed by myosin A and since the binding site of myosin A for pyrophosphate seems to be the same as that for ATP, because pyrophosphate is a competitive inhibitor of myosin A ATPase. Our results showed that one mole of pyrophosphate binds to  $2 \times 10^5$  g of myosin A<sup>7</sup>. KIELLEY and HARRINGTON<sup>8,9</sup> have indicated that in guanidine chloride solution myosin A molecule dissociates into subunits of  $2 \times 10^5$  g molecular weight. As will be described later, both the amount of intrinsic  $\text{Ca}^{2+}$  and the lysine residue attacked specifically by TBS were one mole per  $2 \times 10^5$  g of myosin A. From these results it may be concluded that one subunit of myosin A of molecular weight of  $2 \times 10^5$  g involves one active center. In the following sections the reaction of nucleoside triphosphate and myosin A will be discussed firstly from the kinetic view point and then the effect of structure of the substrate for the enzymic action will be analyzed. The primary and the secondary structure around the myosin A ATPase active site will be described in the subsequent sections.

### **2. Binding of Triphosphate Chain of Substrate to Myosin A:**

#### **Kinetic Study**

It is well known that the enzymic action of myosin A is markedly affected on adding divalent metal ions. Furthermore, myosin A contains  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$ , which cannot be removed by simple washing with deionized water. The weight of the protein per mole of the tightly bound  $\text{Ca}^{2+}$  was  $2 \times 10^5$  g, *i.e.* one mole per mole of subunit of myosin A<sup>10</sup>. In Takaamylase and some proteinases,

the divalent metal ion bound to the enzyme is known to work in keeping the structure for enzyme action. In order to investigate the behavior of the tightly bound  $\text{Ca}^{2+}$  in myosin A, the intrinsic divalent metals were removed from myosin A by blocking of about a half of the SH groups of myosin A by PCMB<sup>11)</sup>. The SH content of myosin A was completely recovered by treatment with cysteine. In the presence of modifiers and under various conditions, the properties of myosin A ATPase treated by PCMB and cysteine were identical to those of the original myosin A which contains the tightly bound  $\text{Ca}^{2+}$ . This indicates that the divalent metal ions tightly bound to myosin A have no connection with myosin A ATPase, though these metal ions are one of the most important factors regulating the inhibition of ATPase and the clearing response of actomyosin on adding high concentration of ATP<sup>11)</sup>.

On the other hand, the effects of the addition of divalent cations such as  $\text{Mg}^{2+}$ ,  $\text{Mn}^{2+}$ ,  $\text{Ca}^{2+}$ ,  $\text{Sr}^{2+}$  and  $\text{Ba}^{2+}$  to the ATPase activity of myosin A were examined by plotting activities of ATPase ( $v$ ) versus ionic radii ( $r$ ). A bell shaped curve was obtained, having its maximum  $v$  at about  $1 \text{ \AA}$  of  $r$ <sup>12)</sup>. The following alternative mechanisms may be possible as the effect of divalent metal ion on myosin A ATPase. The one explanation is that the binding of the substrate to the protein may occur after chelation of divalent ion at the 6-NH<sub>2</sub> end of the purine ring of ATP and terminal phosphate chain of ATP<sup>13)</sup>. The other is that the triphosphate chain of the substrate interacts with the active site of the protein through the divalent metal ion<sup>14)</sup>. To throw the light on this point, we examined the effects of metal ions on the hydrolysis of ATP analogs by myosin A<sup>15)</sup>. The structures of ATP analogs used by us are shown in Table I and the effects of the metal ions on hydrolysis of ATP, ITP, BTP and TP are shown in Fig. 1.

As clearly seen in Fig. 1, the effect of metal ion on the enzymic action of myosin A is insignificantly affected by substituting 6-NH<sub>2</sub> group of purine

TABLE I The Structure of ATP Analogs

Comp.	Structure		
	Base	Sugar	Phosphate
ATP	6-NH <sub>2</sub> -purine	D-ribose	-P-P-P
ITP	6-OH-purine	D-ribose	-P-P-P
dimethyl ATP	6-N(CH <sub>3</sub> ) <sub>2</sub> -purine	D-ribose	-P-P-P
BTP	6-NH <sub>2</sub> -purine	-(CH <sub>2</sub> ) <sub>4</sub> -O-	-P-P-P
RTP		D-ribose	-P-P-P
TP			-P-P-P

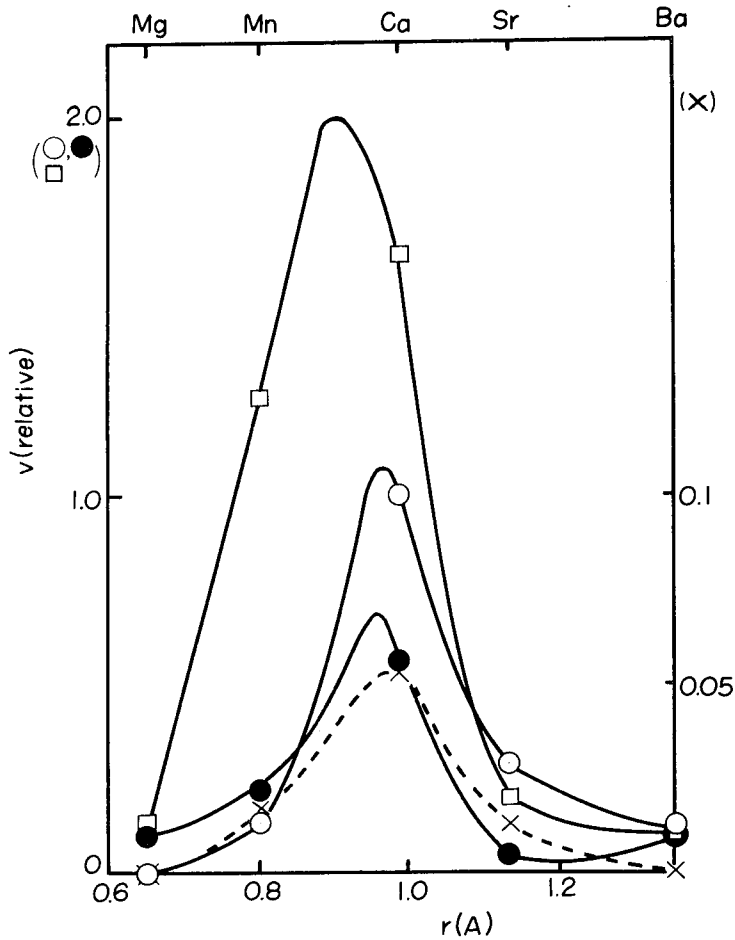


Fig. 1. Effect of divalent cation on myosin A NTPase. The ordinate indicates relative activity, ATPase for the same preparation of myosin A in presence of 5 mM  $\text{Ca}^{2+}$  being defined as 1. The abscissa indicates ionic radius of added divalent cation. The concentrations of substrate and divalent cation were 0.5 and 5 mM, respectively, except for TP, where both concentration of the substrate and divalent cation were 4 mM. Experimental conditions: 0.5 M KCl, pH 7.0, 20°. Substrates: ATP (○), ITP (□), BTP (●) and TP (×).

with OH group and the ribosyl group with  $-(\text{CH}_2)_4\text{-O-}$  or by removing base and ribose from ATP. Thus we must conclude that the triphosphate chain of ATP binds with myosin A by mediation of  $\text{Me}^{2+}$  added.

The pH-activity curve of myosin A ATPase shows complicated features; it shows a maximum and a minimum around neutral pH and then increases



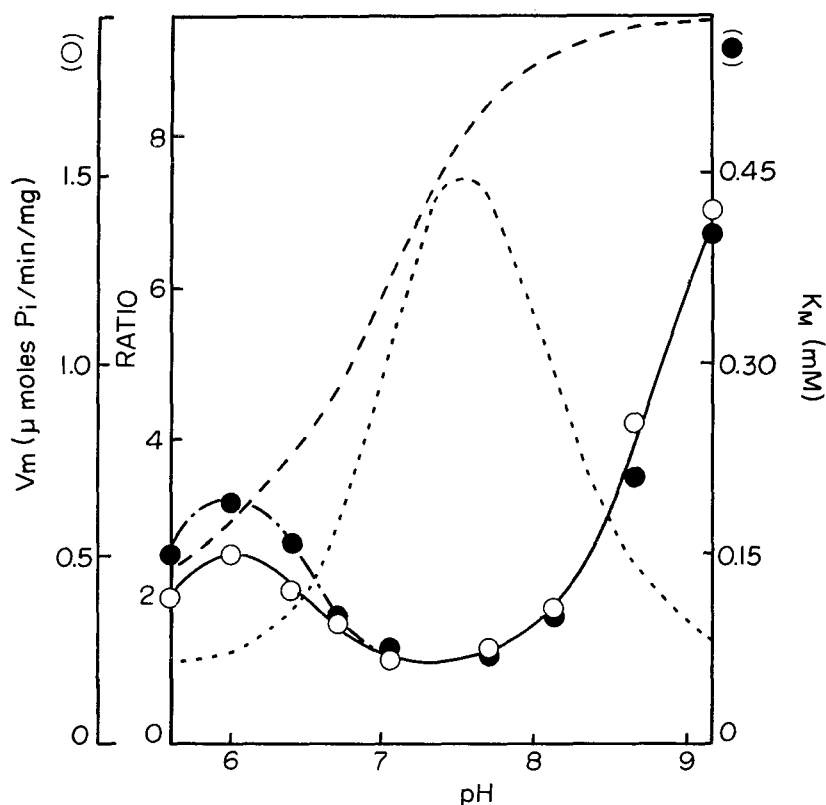


Fig. 2. Effect of pH on  $V_m$  and  $K_M$  of myosin A ATPase. Reaction mixture: 0.6 M KCl, 7 mM  $Ca^{2+}$  and 0.088 mg myosin A/ml. 20°.  $\circ$ ,  $V_m$ ;  $\bullet$ ,  $K_M$ . Broken line is pH-dependence of  $k_2$ . Dotted line is the theoretical curve of pH-dependence of  $1+K$ .

This mechanism is supported by the temperature dependence of  $V_m$  and  $K_M$  of ATPase<sup>19</sup>). At pH 6, where  $E_2S$  is not involved, both the temperature dependence of  $K_M$  and  $V_m$  obeyed the ARRHENIUS equation. On the other hand, at pH 7 where  $E_2S$  is formed, the temperature dependences of  $K_M$  and  $V_m$  were different significantly above and below 10°C from each other. Below 10°C,  $K_M$  decreased slightly and  $V_m$  increased remarkably with increase of temperature. However, above 10°C  $K_M$  decreased significantly and  $V_m$  increased slightly with temperature (Fig. 3). These observations suggest that at high temperatures  $K_M$  is larger and  $V_m$  is smaller than the values which are expected from the observation at lower temperatures because of the formation of  $E_2S$  at higher temperatures. The fact that pH activity curve of myosin A ATPase at 0°C does not show a maximum and a minimum around neutral pH also

indicates that  $E_2S$  is not formed at lower temperatures<sup>19)</sup>. (cf. ref. 20). Thus, the kinetic behavior of ATPase could be explained assuming ATPase as an enzyme containing two MICHAELIS complexes. KRUPKA<sup>21)</sup> have recently explained the pH dependence of chymotrypsin, trypsin and acetylcholinesterase by presence of two MICHAELIS complexes in their enzymic reactions.

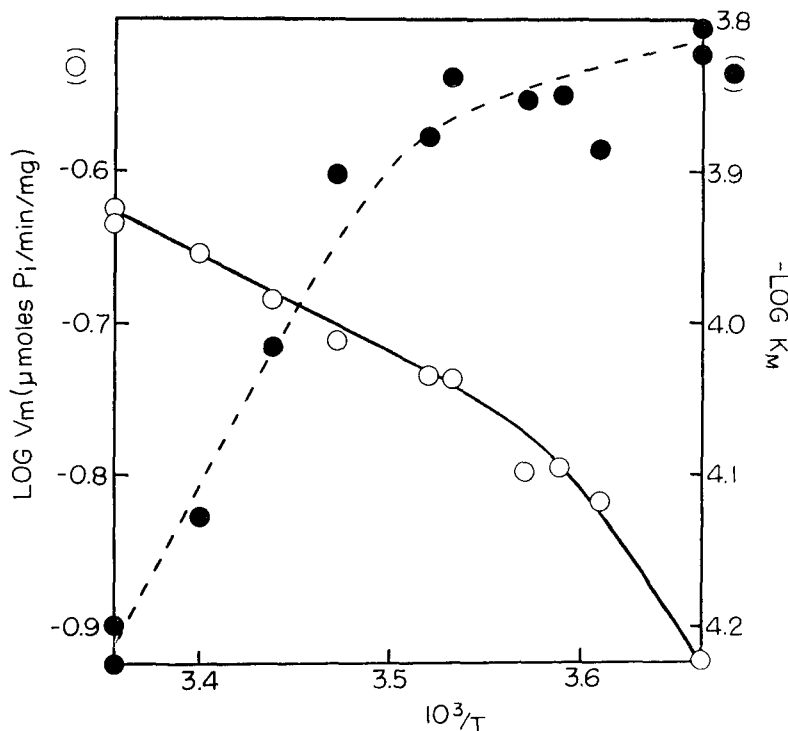


Fig. 3. Effect of temperature on  $V_m$  and  $K_M$  of myosin A ATPase. Reaction mixture: 0.6 M KCl, 7 mM  $C^{2+}$ , 25 mM Tris and 0.11 mg myosin A/ml. pH 7.05. O, log  $V_m$ ; ●,  $-\log K_M$ .

If the mechanism developed above holds for ATPase,  $V_m$  and  $K_M$  of ATPase (at higher temperatures) may be increased and the neutral depression of pH activity curve is eliminated by reagents which hinder the formation of  $E_2S$  but not affect the formation and decomposition of  $E_1S$ . In fact, several reagents are known to exhibit these properties. For example, PCMB activates ATPase at low concentrations though it suppresses the activity at high concentrations<sup>22)</sup>, thus suggesting that a SH group is involved in site 1 as well as in site 2 and the SH group in site 2 is attacked more readily by PCMB than that in site 1. We measured the activity at various pH's after incubation of myosin A with

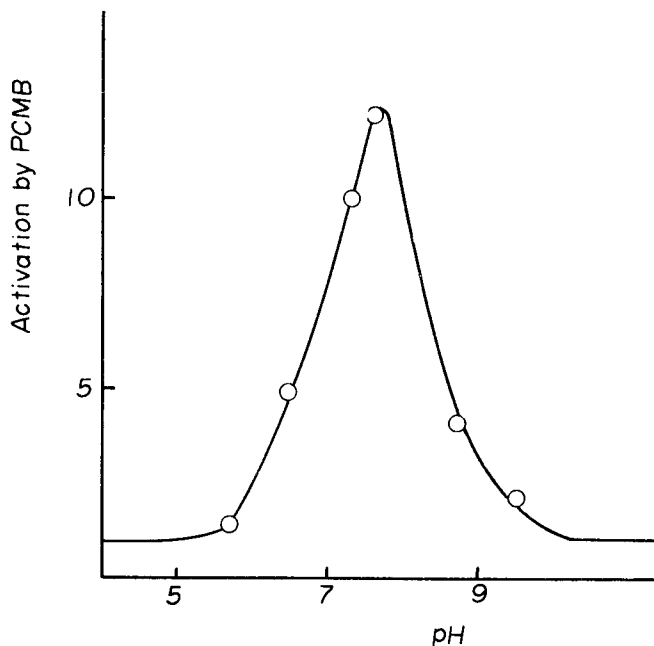


Fig. 4. Dependence on pH of maximum value of degree of activation by PCMB. Binding of PCMB: 0.8 M KCl, pH 6.7, 20°, 30 minutes. Assay of ATPase: 0.6 M KCl, 7 mM Ca<sup>2+</sup>, 1 mM ATP, 20°.

PCMB at a constant pH. The pH dependence of activation by PCMB obtained is shown in Fig. 4<sup>23)</sup>. As expected by the above mechanism where PCMB prevents the formation of the complex E<sub>2</sub>S, the pH dependence of activation by PCMB was almost identical to that of 1 + K given in Fig. 2.

Although PCMB binds to a SH group of myosin A, the mercaptide bond formed is too weak to resist chemical analysis. It has been demonstrated that N-ethylmaleimide and TBS are more suitable reagents for chemical analysis of the structure of the active site. The former is employed by SEKINE and KIELLEY<sup>24)</sup> but we used the latter. TBS shows no absorption at visible region but a clearly yellow color appears when TBS combines to ε-amino group of lysine<sup>25)</sup>. The binding of 1 mole of TBS to 2 × 10<sup>5</sup> g of myosin A occurred rapidly and thereafter the combination increased slowly and linearly with time. The ATPase activity at low ionic strength decreased on the binding of TBS up to 1 mole per 2 × 10<sup>5</sup> g of myosin A and thereafter the activity was kept almost constant<sup>26)</sup>. On the other hand, at high ionic strength the ATPase activity increased with the increment of TBS binding up to 1 mole per subunit of myosin A<sup>10)</sup>. When 1 mole of TBS bound to 2 × 10<sup>5</sup> g of myosin A, the

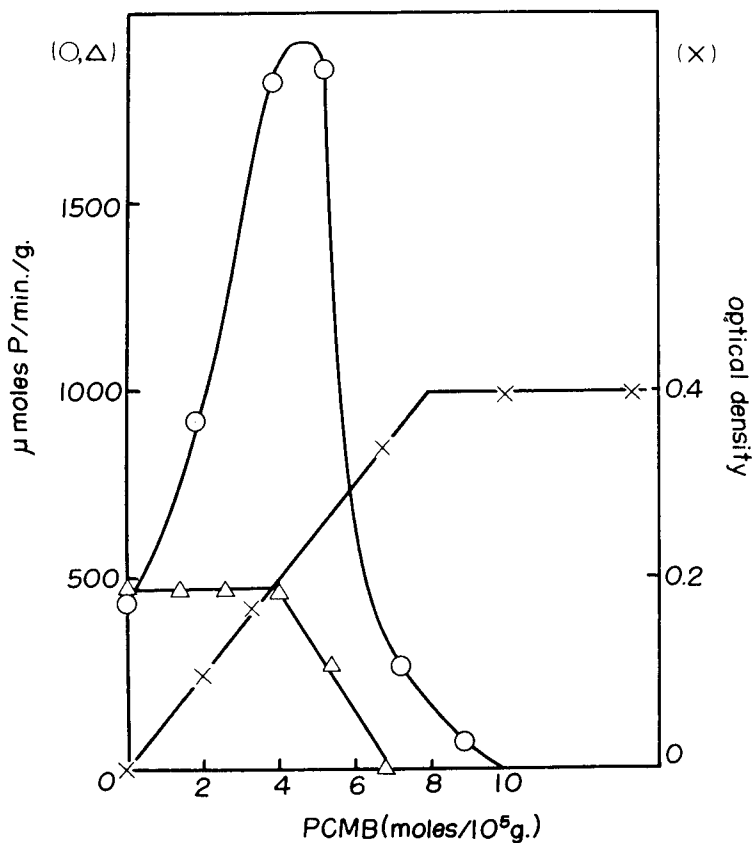


Fig. 5. Effect of PCMB on ATPase of TNP-myosin A. 0.6 M KCl, 7 mM Ca<sup>2+</sup>, 1 mM ATP, pH 7.0, 20°. ○, original myosin A; △, 1.35 mole of TBS/2.1 × 10<sup>5</sup> g of myosin A; ×, spectrophotometric titration of SH group of TNP-myosin A with PCMB.

typical depression of the pH-activity curve around the neutral region and the activation by EDTA and PCMB disappeared (Fig. 5). These results strongly indicate that the formation of E<sub>2</sub>S is hindered by the binding of TBS to one lysine residue in the active site of ATPase.

### 3. Binding of Substrate at Base Moiety

In this section the role of the base moiety of substrate in its reaction with myosin A will be described. The  $K_M$ 's of hydrolysis of natural nucleoside triphosphates have been determined by many workers (*cf.* ref. 14), and the  $K_M$ 's of hydrolysis of ITP, BTP, RTP, dimethyl ATP, TP and ATP have

recently been measured by us<sup>15)</sup>. The decreasing orders of the binding strength of myosin A NTPase obtained from their  $K_M$ 's are ATP, CTP; UTP, GTP, ITP and dimethyl ATP, BTP, RTP, TP. In the preceding section, the equilibrium of  $E_1S \rightleftharpoons E_2S$  was assumed. When the equilibrium of the step  $E_1S \rightleftharpoons E_2S$  is maintained at more right side, the value of  $K_M$  of a NTPase might become smaller and the complicated behavior of a NTPase become more distinguished. The hydrolysis of BTP, RTP and TP, which shows large  $K_M$ , lacked the neutral depression in the pH activity curve and was not activated by PCMB and EDTA (Fig. 6), though ATPase which has small  $K_M$  shows

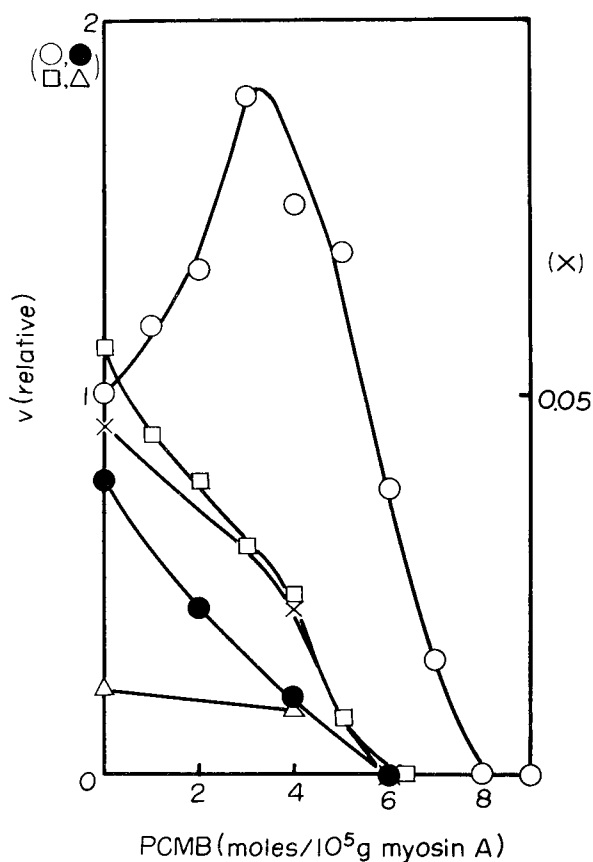


Fig. 6. Effect of PCMB on hydrolysis of ATP analogs. The concentrations of substrate and  $Ca^{2+}$  were 0.5 and 5 mM, respectively, except for TP, where both concentrations of the substrate and  $Ca^{2+}$  were 4 mM. PCMB was incubated with myosin A during 20 min. at 0° before start of the reaction. Experimental conditions: 0.5 M KCl, pH 7.0, 20°. Substrates: ATP (○), ITP (□), dimethyl ATP (△), BTP (●) and TP (×).

the reduced activity at neutral region. The same relation between  $K_M$  and other enzymic properties was also reported on natural NTP.

What group of the base plays an important role in binding of ATP, CTP, GTP, UTP and ITP with myosin A? The formation of  $E_2S$  was observed in the case of ATP and CTP but was not observed for GTP, UTP and ITP (*cf.* ref. 14). From the analysis of infrared absorption of nucleosides, Tsuboi *et al.*<sup>27)</sup> have recently demonstrated that the values of pK of dissociation of  $NH^+$  at position 1 of the base of ATP and CTP are 3.5 and 4.2, respectively, and the values of GTP, UTP and ITP, of which the pH-activity curves lack the neutral depression, are 9.2, 9.45 and 8.8, respectively. Therefore, it may be concluded that only the substrate whose N at position 1 of the base is in dissociated state shows complicated features of the enzymic action, *i.e.* can form the  $E_2S$  complex.

Thus the substrate binds to myosin A at the triphosphate group by mediation of  $Me^{2+}$  as well as at the base part of the substrate. The binding of triphosphate occurs in two ways; the one is an active binding at site 1 which is hydrolyzed into free enzyme and products and the other is an inactive one at site 2 which is not hydrolyzed. The dissociation of  $NH^+$  at position 1 of the base of the substrate determines whether binding of nucleotide at site 2 occurs or not. As the secondary structure of the enzyme protein is closely related to this phenomenon, the molecular explanation of this effect will be mentioned later in relation to the secondary structure of the protein.

#### 4. Relation of Structure of Substrate to Hydrolysis

In the preceding parts we have analyzed the binding of the substrate with myosin A. In this section, the relation of the structure of substrates to their velocity of hydrolysis will be mentioned.

It has recently been reported by us<sup>15)</sup> that the rates of ITPase and of ATPase (under the conditions where the formation of  $E_2S$  is inhibited) are the highest and those of BTP and dimethyl ATP are smaller than them, while those of RTP and TP are the lowest (Fig. 7). This observation clearly indicates that the rate of breakdown of the complex  $E_1S$  depends on the binding of substrate at 6- $NH_2$  or 6-OH group of the base on myosin A. The rate of hydrolysis of BTP was lower than ITP and higher than RTP. Even at high concentrations ribose did not inhibit ATPase. These results suggest that the C-C bonds of  $-(CH_2)_4-$  of BTP permit free rotation, so that when the compound binds to the active site to form  $E_1S$ , there will be a loss of the configurational entropy. In Fig. 7 is shown the effect of pH on the hydrolysis of various triphosphates by myosin A. The increase in rate of breakdown of the complex

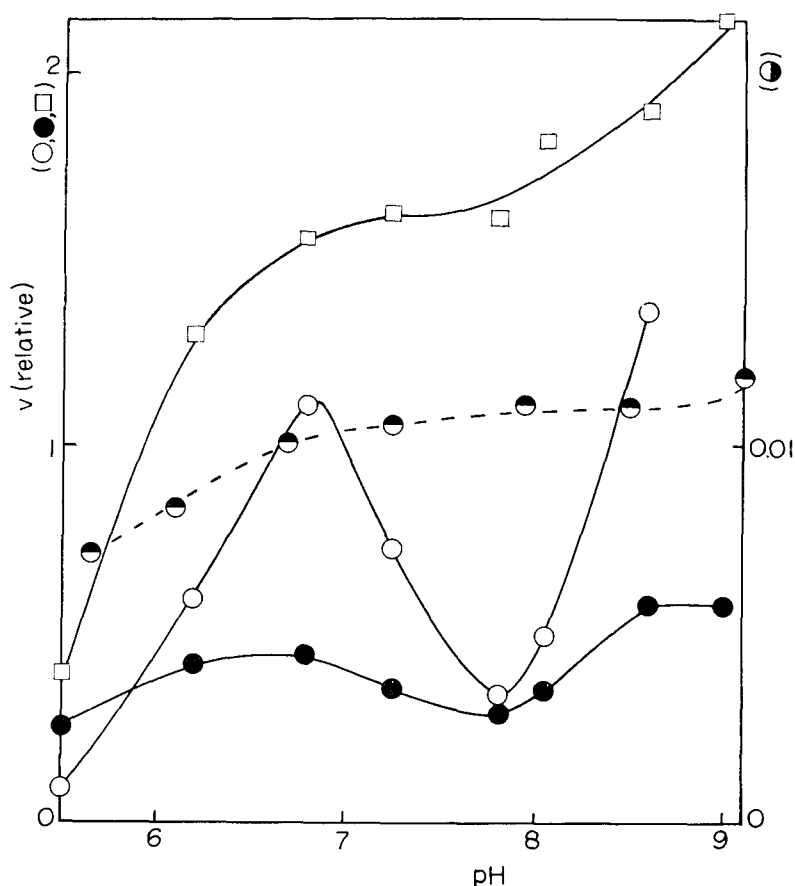
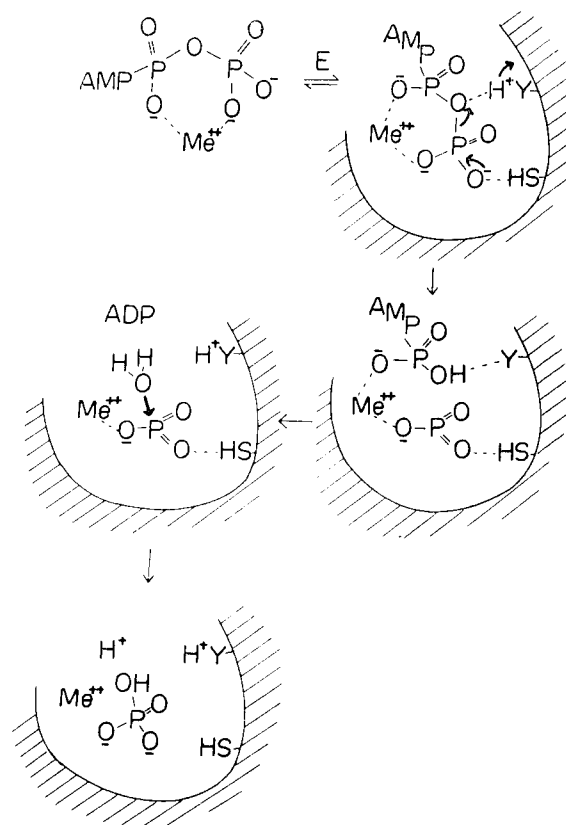


Fig. 7. pH dependence of myosin A NTPase. Tris-maleate buffer (25 mM) was used in the range of pH from 5.5 to 7.8 and Tris-HCl buffer (25 mM) in the range of pH from 7.8 to 9.1. The concentrations of the substrate and  $\text{Ca}^{2+}$  were 0.5 and 5 mM, respectively. Experimental conditions: 0.5 M KCl, 20°. Substrates: ATP (○), ITP (□), BTP (●) and RTP (◐).

$E_1S$  with increasing pH clearly depends on the binding of the substrate at the 6-position of the base on myosin A. However, the binding of adenine with myosin A is rather weak, since ATPase was not inhibited by the addition of adenine even when molar concentration of adenine was 160 times as high as that of ATP<sup>(15)</sup>. Therefore, we have assumed that the enzyme binds with the substrate (in complex  $E_1S$ ) at the 6-position of the base by a hydrogen bonding and the difference in  $K_M$  of NTPase is mainly determined by the equilibrium constant of the reaction  $E_1S \rightleftharpoons E_2S$ .

The rate of hydrolysis of RTP, in which the base of NTP is absent, is almost independent of pH, thus suggesting that the dissociation state of the catalytic group which participates directly on the hydrolysis of pyrophosphate bond does unappreciably change in the range of pH from 5.5 to 9. The slight dependence on pH of hydrolysis of BTP, of which base interaction with myosin A is rather weak, also supports this view. The SH group satisfies this requirement and it seems to be involved in the catalytic state, since myosin A ATPase is inhibited by the addition of a large amount of SH reagent as mentioned above. Modifying somewhat the mechanism<sup>28)</sup> proposed for the hydrolysis of pyrophosphate bond by pyrophosphatase and designating another group in the site as YH<sup>+</sup>, the reaction scheme of ATPase might be proposed as shown in Fig. 8. From the pH dependence of hydrolytic reaction of ATP and ITP and the photooxydation of myosin A ATPase, several workers<sup>29,30,31)</sup>



**Fig. 8.** A tentative scheme of cleavage of pyrophosphate bond catalyzed by myosin A.

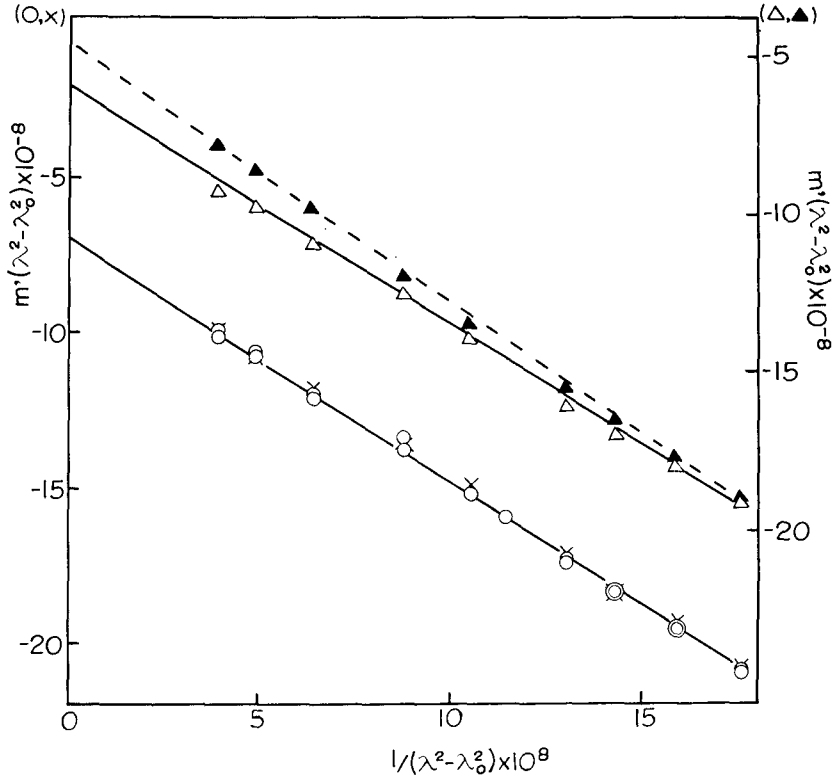


on its binding with the substrate (ATP) or the competitive inhibitor ( $PP_i$ ). Analyses by light scattering, viscosity and ultracentrifugal techniques are usually used as method for approach in this field. The change in size and shape of myosin A molecule on adding ATP or  $PP_i$  cannot be recognized by these methods, though the structure of the main components of myosin B composed of myosin A and actin becomes loose on adding ATP or  $PP_i$ <sup>39</sup>.

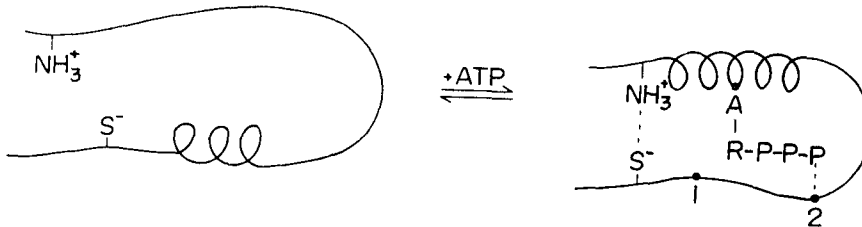
However, our kinetic experiments on denaturation of myosin A showed that the heat inactivation of myosin A ATPase is suppressed on the addition of  $PP_i$ <sup>40</sup>. This is an indirect evidence for some modification in the secondary structure of myosin A molecule on its binding with  $PP_i$ . Therefore, the structural changes of myosin A on its binding with ATP or  $PP_i$  were investigated by measuring properties related more directly and sensitively to the secondary and tertiary structures of myosin A.

The optical rotatory dispersion of myosin A was measured and the excess right-handed helical content of myosin A, estimated by the method of DOTY from the  $b_0$  term of the MOFFITT-YANG plot, was found to be about 60%. As shown in Fig. 10, the helical content of myosin A decreased by several percent on adding  $Mg^{2+}$ - $PP_i$ , but increased by several percent on adding  $Mg^{2+}$ -ATP, though no change was detected on the addition of  $Mg^{2+}$ -ADP which is the product of ATPase<sup>41</sup>. Two percent increment in the helical content of myosin A means the incorporation in helical structure of 36 amino acid residues per  $2 \times 10^5$  g of myosin A. As the decrease in entropy of the transformation from random coil to helix is about 4 *e.u.* per amino acid, this change accompanies the decrease in entropy of 144 *e.u.* per subunit of myosin A. Thus, the slight change of helical content of myosin A on adding ATP or  $PP_i$  accompanies a large change in entropy.

As mentioned above, the pH dependence of the equilibrium constant of the reaction  $E_1S \rightleftharpoons E_2S$  is given by a bell-shaped curve, having its maximum at pH 7.5; this indicates that  $E_2S$  complex occurs when the salt linkage between  $-NH_3^+$  and  $-S^-$  is constructed. In fact, TBS and PCMB, which prevent the formation of  $E_2S$ , bind specifically with  $-NH_2$  group of lysine and  $-SH$  group of cysteine, respectively. From these results, together with the conformation change of active site on its binding with ATP, the following picture might be proposed as a model of the ATPase active site, though it is undoubtedly oversimplified (Fig. 11). An  $NH_2$  group and a SH group are located near the active site. When the base of ATP binds at position 1 with myosin A, the helical structure will be constructed near the binding site. On the other hand, on binding the TP chain of ATP the helical structure will be melt and then the salt linkage between  $-NH_3^+$  and  $-S^-$  can be formed. When the structure of



**Fig. 10.** MOFFITT-YANG plot of dispersion data in presence of  $Mg^{2+}$ -ADP and  $Mg^{2+}$ -ATP. 0.6 M KCl, 20 mM  $Mg^{2+}$ , 3.81 mg myosin A/ml, pH 7.0, 13°. O, control; ×, 3 mM ADP. △, control; ▲, 3 mM ATP.

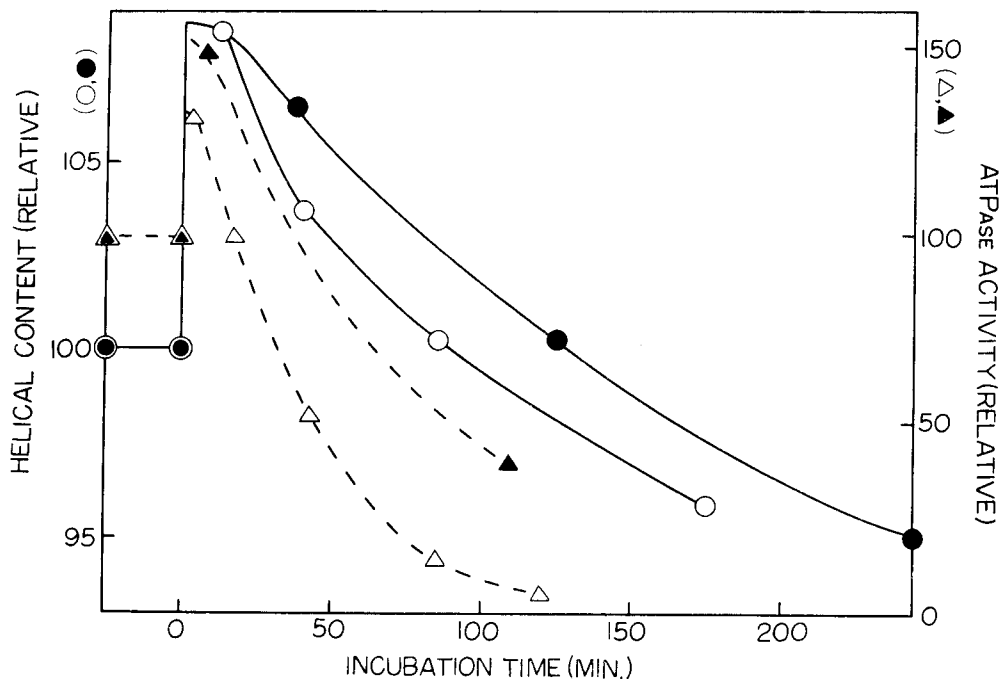


**Fig. 11.** A formation of active site by conformation change and salt-bridge.

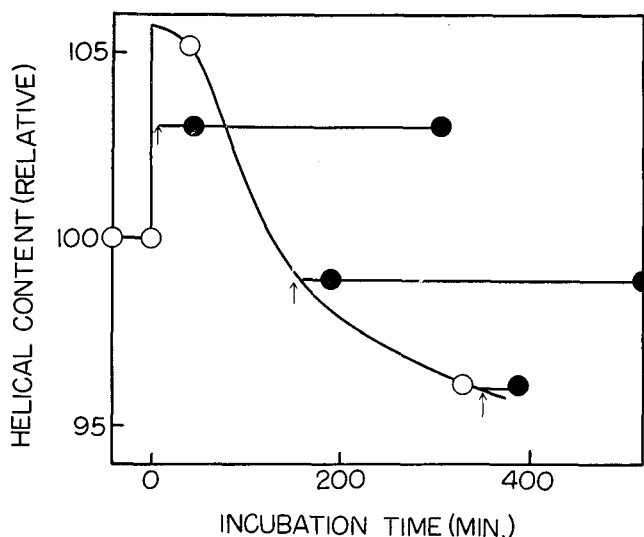
the active site is constructed in this manner, the TP chain of ATP can bind with both site 1 and 2, and myosin A ATPase shows its characteristic features as discussed above.

In the above discussion, PCMB and TBS are considered to affect ATPase by simply breaking the salt linkage of  $-NH_3^+ \cdots S^-$ . However, the helical structure

around the active site is changed by binding with these reagents<sup>42</sup>). It has been demonstrated that 4 moles PCMB/10<sup>5</sup> g increases the helical content by a few percent and induces a remarkable activation of ATPase, while 8 moles PCMB/10<sup>5</sup> g decreases in the helical content by a few percent and inhibits ATPase completely. The changes of essentially a similar nature were also observed on the addition of organic solvent to the enzyme<sup>6,42</sup>). For example, immediately after the addition of dioxane the helical content increased by several percent of the control value and the ATPase activity increased significantly. 100–200 min. after the addition of dioxane the helical content decreased by several percent and the activity disappeared almost completely. That is, a slight increase and decrease in helical content have been accompanied, respectively, by the remarkable increase and decrease in ATPase activity (Fig. 12). The



**Fig. 12.** Time-courses of changes in helical content (O, ●) and ATPase activity (△, ▲) after addition of dioxane. 0.6 M KCl, pH 7.0, 20°. Helical content was estimated from  $b_0$  term and ATPase activity measured in 7 mM Ca<sup>2+</sup>, 0.5 mM ATP and 10 mM Tris-maleate buffer. Helical content: O, 10 volume % dioxane, 4.65 mg myosin A/ml; ●, 8 volume % purified dioxane, 3.62 mg myosin A/ml. ATPase activity: △, 10 volume % dioxane, 0.2 mg myosin A/ml; ▲, 8 volume % purified dioxane, 0.2 mg myosin A/ml.



**Fig. 13.** Effect of  $PP_i$  on time-course of change in helical content in presence of dioxane. 0.6 M KCl, 6.14 mg myosin A/ml. pH 7.0, 20°. 8% purified dioxane was added at time 0. ○, in absence of  $PP_i$ ; ●, in presence of 1 mM  $Mg^{2+}\text{-PP}_i$ . Arrow indicates addition of  $Mg^{2+}\text{-PP}_i$ .

properties of activation by dioxane were very similar to those of activation by PCMB. Furthermore, on adding dioxane to myosin A in the presence of  $PP_i$ , no change in the helical content was observed (Fig. 13). A similar result was obtained on the alkali-inactivated myosin A. From these facts it may be deduced that these changes in helical content take place specifically and locally around the active site of the enzyme. The apparently close relation between a large change in ATPase activity and a slight change in helical content by dioxane and PCMB suggests that the effects of these reagents on the enzyme activity cannot be attributed simply to the breakdown of the salt linkage as assumed above.

The secondary structure around the ATPase active site is particularly sensitive also to other treatments. The helical content and viscosity of myosin A reduced conspicuously in a narrow range of the concentration of salt, for example LiBr<sup>(43)</sup>. The temperature dependence of the decrease in helical content by adding LiBr obeyed the SCHELLMAN equation for the transformation of helix to random coil. The change in the structure of the myosin A molecule induced by the salt is apparently reversible. However, ATPase activity is inhibited irreversibly even by the range of low concentration of LiBr where the helical content of myosin A remained almost unaffected. A similar change was also observed on adding various salts (except for KCl and NaCl)<sup>(43)</sup> and on the heat,

acid and alkaline denaturation of myosin A. When the helical content reduced by a few percent on the heat or pH denaturation, ATPase activity diminished completely<sup>17)</sup>.

Thus various reagents seem to induce remarkable change in ATPase activity by changing slightly and locally the secondary structure of myosin A around the active site. However, several recent observations suggested that changes in the conformation of myosin A on adding ATP or  $PP_i$  is rather profound and extends to the protein molecule as a whole. For example, in NaCl solution muscle cannot contract and the binding of ATP at site 2 cannot occur in NaCl solution. However, the helical content by several percent on adding ATP was observed in NaCl as in KCl solution<sup>41)</sup>.

Therefore, the following experiments were made to clarify the significance of these differences in KCl and NaCl not only on the structural change of myosin A caused by binding with the substrate but also on molecular mechanism of muscle contraction in general<sup>41)</sup>. Fig. 14 shows the titration curves of tyrosyl residue by measuring ultraviolet absorption at  $259\text{ m}\mu$  of myosin A at different pH's. From this result the number of tyrosine which is embedded in the protein

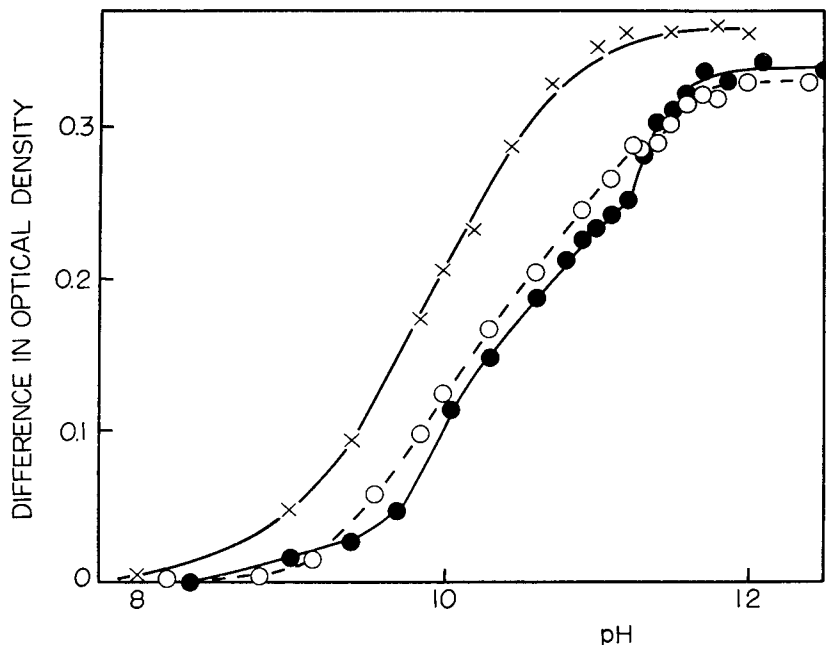
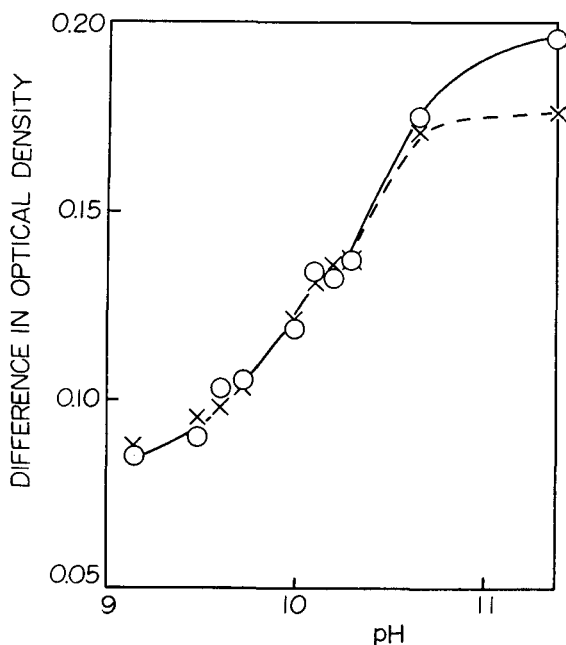


Fig. 14. Spectrophotometric titration of myosin A. 0.5 M KCl, 50 mM piperidine buffer, 1 mg myosin A/ml, 25°. O, control; x, 1.2 M urea-5 M guanidine HCl. The line is theoretical one for dissociation of a single group whose pK is 9.85. ●, 0.5 mM  $Mg^{2+}$ - $PP_i$ .

can be estimated. When the secondary structure of protein was destroyed perfectly by adding urea-guanidine, the titration curve was given by a simple dissociation curve, *i.e.* all the tyrosyl residues were normal. In 0.5 M KCl myosin A showed about 3 moles of abnormal tyrosine per  $10^5$  g protein. On adding  $PP_i$  or ATP the number of abnormal tyrosine increased to about 6 moles per  $10^5$  g protein. Thus, on adding the substrate about 6 moles tyrosine per  $2 \times 10^5$  g are buried in the protein structure from the surface. It is very difficult to explain this observation by the local change of the secondary structure around the active site. In NaCl solution where the particular structure for contraction is not constructed, the significant change in dissociation state of tyrosine was not observed on adding ATP. Accordingly, it may be concluded that the structure of a large part of myosin A may contribute to the formation of the conformation for ATPase active site and for muscle contraction.

A situation similar to this has been observed with SH-group<sup>44</sup>. The ATPase activity was inhibited competitively by nitroprusside. However, the amount of SH group titratable with nitroprusside was unaffected by the addition of so large amount of ATP that the competitive inhibition by nitroprusside could be



**Fig. 15.** Effect of ATP on nitroprusside color development of myosin A. Light pass, 1 cm. O, 1.72 mg myosin A/ml. plus 45 mM nitroprusside, 0.3 M KCl at 0°. X, 1.72 mg myosin A/ml. plus 2 mM ATP, 5 mM  $Mg^{2+}$  and 45 mM nitroprusside, 0.3 M KCl at 0°.

removed (Fig. 15). This may suggest that when ATP binds to an untitratable SH group of myosin A, the conformation of myosin A is changed and the SH group becomes to be titrated by nitroprusside.

Thus, the secondary and tertiary structure of the myosin A molecule is changed rather extensively by various reagents, especially, by the binding of the substrate, though the relation between the change in the secondary structure and the enzymic activity of myosin A is not obvious. These marked dynamic behaviors of myosin A molecule may be important for elucidating the molecular mechanism of not only ATPase activity but also the physiological function of myosin A, since the interaction of myosin A and ATP seems to be an essential step in muscle contraction<sup>5)</sup>.

## 6. Primary Structure of Active Site

In this section, the chemical structure of the active site of myosin A will be described. PCMB is an unsuitable reagent for the chemical analysis of the active site, since the mercaptide bond formed is too weak to resist acid hydrolysis of peptide bond to obtain subfragments. A reagent which binds specifically and stoichiometrically with the active site of myosin A through a covalent bond must be used. Recently KIELLEY and SEKINE<sup>24)</sup> are studying structure of site 2 as termed by us using N-ethylmaleimide as the reagent.

Determination of the amino acid sequence around the site attacked by TBS is currently being in progress in our laboratory, as the binding of 1 mole of TBS to  $2 \times 10^5$  g of myosin A exhibits very specific and stoichiometric effect on the myosin A ATPase activity. ATPase activity of TNP-myosin A was not activated by the addition of EDTA or PCMB and lacks the characteristic depression at the neutral region of the pH activity curve, as mentioned above. As the amount of  $\epsilon$ -TNP-lysine obtained from the acid hydrolyzate of TNP-myosin A was in good agreement with that estimated by the absorption of TNP-myosin A, it was concluded that TBS binds specifically and stoichiometrically to the amino group of one lysyl residue in one myosin A subunit<sup>26)</sup>. TNP-myosin A was partially hydrolyzed by subtilisin and  $\epsilon$ -TNP-peptides produced were absorbed on talc column and after removal of TNP the peptides were isolated by column chromatograph on Dowex  $50 \times 2$ . The primary structure around the lysine residue near the active site has been shown to be: Ser. (Gly. Ala. Ser. Gly.) (Glu. TNP-Lys.) (Asp. Leu. Glu.)<sup>45)</sup>.

## Conclusion

The mechanisms of both the binding to myosin A and the hydrolysis by myosin A of the substrate were clarified firstly by the kinetic analysis and then

by using ATP analogs. In the later sections, the structure of active site of myosin A ATPase was discussed from changes in secondary structure of myosin A by its combination with the substrate and the modifiers and from the determination of the sequence of amino acids around the lysine involved in the active site of ATPase.

However, as shown above, many problems still remain to be clarified. Especially, the perfect determination of the primary structure of the active site and the clarification of the relation between the secondary structure of myosin A molecule and the activity as ATPase seem to be the most important points for further investigations.

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