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KINETICS OF STEADY STATE ATPase ACTIVITY AND RIGOR COMPLEX FORMATION OF ACTO-HEAVY MEROMYOSIN

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SUMMARY

1. Actin and heavy meromyosin, initially mixed in a Mg–ATP solution, began to form the rigor complex slowly after ATP in the solution had been completely hydrolyzed.

2. This was because the heavy meromyosin–product complex formed via ATP hydrolysis was almost completely dissociated from actin even in the absence of ATP and as soon as this heavy meromyosin–product complex was decomposed, the heavy meromyosin combined with actin forming the rigor complex.

3. Linear plots were obtained when the reciprocal of the excess rate of the actin-accelerated rigor complex formation was plotted against the reciprocal of the added actin concentration as similar with those made on the steady acto-heavy meromyosin ATPase.

4. The V of the rigor complex formation process was about 1/5 of that of the steady acto-heavy meromyosin ATPase activity, showing that the actomyosin ATPase activity could not be explained merely by the actin-accelerated decomposition of the heavy meromyosin–product complex.

5. The same analyses were carried out on myosin subfragment 1.

6. Our results could be explained by considering the two non-identical active sites of myosin, and we propose the following scheme for the actomyosin ATPase.

7. Actin accelerates the rate-limiting bond hydrolysis in the ATPase occurring at one active site of myosin, as well as the rate-limiting decomposition of the heavy meromyosin–product complex formed at another site.

INTRODUCTION

The myosin–product complex is formed as a rate-limiting intermediate in the myosin ATPase activity [1–3]. The myosin–product complex formed via ATP

Abbreviations: myosin*–ADP–P_i; the myosin–product complex formed via ATP hydrolysis.

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hydrolysis (myosin*-ADP-P_i) is different from the myosin–product complex (myosin–ADP-P_i) formed by simply being mixed with the products (ADP and/or P_i) [4–8].

Recent investigations on the reaction mechanism of the myosin ATPase have concentrated on elucidating the biochemical and biophysical properties of myosin*–ADP-P_i [9–11]. The function of myosin*–ADP-P_i is a problem to be solved in the actin-accelerated Mg–ATPase activity of myosin, since the actomyosin ATPase activity has been believed to be the biochemical manifestation of muscle contraction in vitro.

A reaction scheme for the actomyosin ATPase was proposed by Taylor [2] and Lymn and Taylor [12]:

$$\text{A–myosin}+\text{ATP} \rightleftharpoons \text{A}+\text{myosin–ATP} \xrightarrow{\text{fast}} \text{A}+\text{myosin*–ADP–P}_i$$
$$\rightleftharpoons \text{A–myosin*–ADP–P}_i \longrightarrow \text{A–myosin}+\text{ADP}+\text{P}_i$$

where A is actin. The equilibrium between actin and myosin*–ADP-P_i was introduced because the acto-heavy meromyosin ATPase activity obeys the double-reciprocal plots of the steady ATPase rate versus actin concentration [13].

Tonomura and his collaborators proposed another scheme [3, 14, 15]:

$$\text{A–myosin}+\text{ATP} \rightleftharpoons \text{A–myosin–ATP}$$
$$\rightleftharpoons \text{A–myosin(ADPP}_i\text{)} \xrightarrow{(1)} \text{A–myosin}+\text{ADP}+\text{P}_i$$
$$\text{A}+\text{myosin*–ADP–P}_i \xrightarrow{(2)}$$

where myosin(ADPP_i) is the "phosphorylated myosin", tentatively introduced by them to explain the "extra burst" of P_i liberation by myosin at low Mg^{2+} concentration as well as by actomyosin when mixed with Mg–ATP. In this scheme the main route in the ATPase activity is assumed to be via Process (1).

A myosin molecule has two heads, on each of which an active site is located [16–19]. It is still undecided whether the two active sites of myosin are identical or not.

In the former scheme the two sites are assumed to be identical, whereas in the latter scheme it is assumed that the two sites are not identical and only one site operates during the actomyosin ATP hydrolysis.

We found that the process of the actin-accelerated decomposition of myosin*–ADP-P_i could be measured by following the formation of the rigor complex of actin and heavy meromyosin after the depletion of ATP in the solution. We found that none of the above schemes could explain the results obtained by analysing the formation process by the double-reciprocal plots in comparison with the steady acto–heavy meromyosin ATPase. One possible way to explain the results is to consider that the two active sites have non-identical functions in the actomyosin ATPase as will be shown later, and an alternative scheme for the actomyosin ATPase will be proposed in the present paper.

MATERIALS AND METHODS

Actin was extracted from acetone-dried muscle powder of rabbit skeletal muscle at 0 °C to avoid contamination with tropomyosin and troponin [20]. Actin was purified by polymerization-depolymerization cycles. Tryptic heavy meromyosin was prepared by the method of Lowey and Cohen [21]. The fraction that was precipitated between 45 and 55% saturation of ammonium sulphate was used. Subfragment 1 was prepared by tryptic digestion of heavy meromyosin in 0.1 M KCl and 20 mM Tris–HCl buffer (pH 7.6) at 0 °C by using trypsin in 1 : 20 weight ratio to heavy meromyosin. The mixture was dialyzed overnight at 4 °C against 10 mM phosphate buffer (pH 7). The KCl concentration of the medium decreased to about 0.01 M after the dialysis. The digestion was stopped by adding trypsin inhibitor at a 2 : 1 weight ratio of inhibitor to trypsin. The resulting digest was applied to a Sephadex G-200 column eluted with 0.15 M KCl–10 mM Tris–HCl buffer (pH 7.6), and fractions containing subfragment 1 were combined.

p-Nitrothiophenylated heavy meromyosin [22] was prepared by incubation of 20 mg/ml heavy meromyosin with 3 mM *p*-nitrothiophenol for 30 h in the presence of 1.0 M KCl, 20 mM $MgCl_2$, 2 mM ATP and 0.1 M Tris–maleate buffer (pH 6.5) at 0 °C. To keep the ATP concentration nearly constant, a proper amount of ATP was added to the reaction mixture at suitable intervals. The resulting compound was isolated by gel filtration through a column of Sephadex G-25 at 4 °C. Then the compound was incubated for 5 h at 25 °C to remove unstably bound *p*-nitrothiophenol. The free *p*-nitrothiophenol liberated was removed by dialysis against 60 mM KCl and 10 mM Tris–maleate buffer (pH 7) at 0 °C for 48 h. The amount of *p*-nitrothiophenol in the compound was determined by measuring the absorbance at 412 nm ($\varepsilon = 1.36 \cdot 10^4$ cm^2/M) after heating the compound for 10 min in 0.1 M NaOH, followed by the removal of precipitated protein [23].

Protein concentrations of actin and heavy meromyosin were determined by the biuret reaction. The subfragment 1 concentration was determined from absorbance at 280 nm ($\varepsilon = 770$ cm^2/g for subfragment 1) [24].

The ATPase activities of acto–heavy meromyosin and subfragment 1 were measured at 20 °C. P_i was determined by the Martin and Doty procedure [25].

Changes in the scattered light from protein solutions were measured at 20 °C at an angle of 90° from the incident beam. An actin solution containing ATP (10 ml) was mixed with 10 ml of heavy meromyosin or subfragment 1 solution by a simple mixing device composed of two syringes. Alternatively a heavy meromyosin solution (7 ml) was first mixed with an ATP solution (7 ml), and after 5 s, 7 ml of actin solution was mixed with the ATP–heavy meromyosin mixture by a mixing device composed of three syringes. The molecular weights of heavy meromyosin and subfragment 1 were taken as $3.4 \cdot 10^5$ (ref. 21) and $1 \cdot 10^4$ (ref. 26).

The ATP, trypsin and soybean trypsin inhibitor were purchased from Sigma Chemical Co. All other reagents were of analytical grade from Nakarai Chemical Co. (Kyoto).

RESULTS

Actin and heavy meromyosin are dissociated in a Mg–ATP solution, whereas in the absence of ATP (and even in the presence of ADP) they bind firmly forming

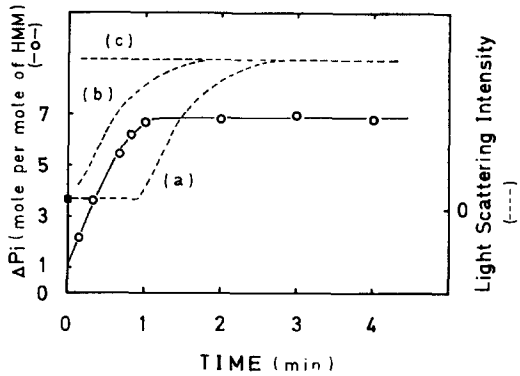


Fig. 1. Correlation of phosphate liberation (○–○) and time course of increase in light-scattering intensity (- - -) after adding heavy meromyosin (HMM) to a mixture of ATP and actin. 1 mg/ml heavy meromyosin, 0.5 mg/ml actin, 0.3 M KCl, 10 mM Tris–maleate (pH 7), 1 mM $MgCl_2$ at 20 °C. a. (○–○), 0.021 mM ATP (= 7× [heavy meromyosin]); b. 0.003 mM ATP (= [heavy meromyosin]); c. 0.01 mM ADP and 0.01 mM P_i. (■), light-scattering intensity from actin solution without heavy meromyosin. HMM, heavy meromyosin.

the "rigor complex" [27]. The light-scattering intensity from the solution containing the rigor complex is appreciably larger than that of the dissociated acto–heavy meromyosin solution [28], which is nearly identical to that of the actin solution without heavy meromyosin (see Fig. 1). Hence, we can follow the process of the rigor complex formation by measuring changes in the light-scattering intensity.

We found that actin and heavy meromyosin, initially mixed in an ATP solution, began to form the rigor complex after ATP had been almost completely hydrolyzed by heavy meromyosin (Curve (a) in Fig. 1). It took about 1 min for the retarded rigor complex formation to be completed after its initiation. On the other hand, the formation of the rigor complex was completed immediately after the mixing of heavy meromyosin with actin in the solution of Mg–ADP and inorganic phosphate (P_i), as well as in the absence of both ATP and ADP (Curve (c) in Fig. 1). In the experiment shown by Curve (a) in Fig. 1, the solution contained 7 times more ATP compared to molar heavy meromyosin, and when error in the P_i determination is taken into consideration, the concentration of ATP, if present, at the complete consumption of added ATP, may be much smaller than 1/10 of that of heavy meromyosin. This means that the retarded rigor complex formation is not due to the effect of the minute amount of ATP present at the end of ATP consumption, since, if this is merely so, approx. 9/10 of heavy meromyosin would not bind ATP and therefore would form the rigor complex at the end of ATP consumption, contrary to the observation of the Curve (a) in Fig. 1. Since a rate-limiting intermediate in the ATP hydrolysis by heavy meromyosin is the heavy meromyosin*–ADP–P_i complex, the retarded rigor complex formation may be interpreted as representing an almost completely dissociation of the heavy meromyosin*–ADP–P_i and actin even in the absence of ATP, and moreover, that the rigor complex is formed as soon as the heavy meromyosin*–ADP–P_i complex is decomposed. (Note that the size of the pre-steady state burst of P_i liberation by heavy meromyosin is about one mole per mole of the enzyme (Fig. 1). The same size was obtained even at

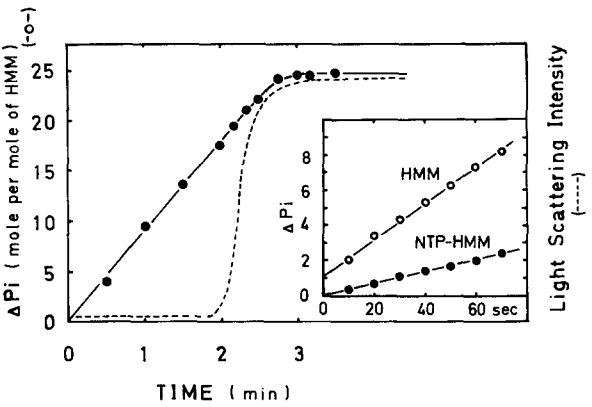


Fig. 2. Correlation of phosphate liberation (●–●) and time course of increase in light-scattering intensity (- - -) after adding nitrothiophenylated heavy meromyosin (NTP-HMM) to a mixture of ATP and actin. 0.4 mg/ml nitrothiophenylated heavy meromyosin, 1 mg/ml actin, 0.1 M KCl, 10 mM Tris–maleate (pH 7), 1 mM $MgCl_2$ and 0.03 mM ATP at 20 °C. The insert in the figure shows the ATPase activities of heavy meromyosin (HMM) and nitrothiophenylated heavy meromyosin. 2 mg/ml protein, 0.1 M KCl, 10 mM Tris–maleate (pH 7), 1 mM $MgCl_2$ and 0.5 mM ATP at 20 °C. Note that the size of the pre-steady state burst of P_i liberation of heavy meromyosin and nitrothiophenylated heavy meromyosin are 1 and 0 mole per mole of protein.

higher ATP concentrations. Therefore, the molar ratio of the product to heavy meromyosin in the heavy meromyosin*–ADP–P_i complex is one [3]. We obtained the same burst size with heavy meromyosin lyophilized in 0.2 M sucrose for storage, freshly prepared myosin and glycerinated myosin.)

Furthermore, the following three experiments (Curve (b) in Fig. 1, and Figs 2 and 3) verify the above interpretation.

5 s after mixing heavy meromyosin with an equimolar ATP solution, actin was added to the mixture (heavy meromyosin+ATP). Immediately after the mixing, the rigor complex began to form, and the rate was slow as in the case of 7 times more ATP compared to molar heavy meromyosin (Curve (b) in Fig. 1). Most of the heavy mero-

TABLE I

ACTIN ACTIVATION OF THE HEAVY MEROMYOSIN AND NITROTHIOPHENYLATED HEAVY MEROMYOSIN Mg–ATPase ACTIVITIES

Conditions: 1 mg/ml actin, 0.1 M KCl, 10 mM Tris–maleate (pH 7) and 1 mM $MgCl_2$ at 20 °C. ATP concentration was 0.1 mM in samples without actin and 0.5 mM in samples with actin. Heavy meromyosin concentration was 2 mg/ml in samples without actin and 0.4 mg/ml in samples with actin.

Enzyme	ATPase (μmoles/mg min)		Actin activation
	Without actin	With actin	
Heavy meromyosin	0.024	0.174	×7.25
Nitrothiophenylated heavy meromyosin	0.008	0.024	×3

myosin molecules become heavy meromyosin*–ADP–P_i immediately after the mixing with an equimolar ATP [29] in this experimental condition. Hence, this result shows that no rigor complex is formed between heavy meromyosin*–ADP–P_i and actin.

One mole of *p*-nitrothiophenol which is incorporated into one mole of heavy meromyosin via a covalent bond, inactivates selectively one of the two active sites of heavy meromyosin, which forms heavy meromyosin*–ADP–P_i (refs. 22, 23 and Takehara, S. and Tawada, K., in preparation). The other, unmodified, active site of nitrothiophenylated heavy meromyosin catalyzes ATP hydrolysis without showing the pre-steady state burst of P_i liberation; i.e., the rate-limiting intermediate at this site is myosin–ATP, and this ATPase activity is activated by actin (the insert in Fig. 2 and Table I). The nitrothiophenylated heavy meromyosin and actin, mixed in a Mg–ATP solution, began to form the rigor complex before ATP in the solution was completely hydrolyzed and the complex formation completed simultaneously at the end of the entire ATP consumption (Fig. 2). This experiment shows that the retarded rigor complex formation does not occur in the absence of the generation of heavy meromyosin*–ADP–P_i.

From the above experiments we can conclude that (i) heavy meromyosin*–ADP–P_i and actin are almost completely dissociated even in the absence of ATP; (ii) as soon as heavy meromyosin*–ADP–P_i is decomposed, the heavy meromyosin molecule forms the rigor complex since heavy meromyosin and actin, mixed in an Mg–ADP solution, form the rigor complex instantaneously; and (iii) the rate-limiting step in this process may be the decomposition of heavy meromyosin*–ADP–P_i. The slow rate of the retarded rigor complex formation is due to the slow decomposition rate of heavy meromyosin*–ADP–P_i [17, 29].

Since the increase in the light-scattering intensity is strictly proportional to the amount of the rigor complex [30, 31], we can quantify the time course of the rigor complex formation by measuring changes in the light-scattering intensity. The retarded rigor complex formation followed a simple exponential process while the molar concentration of heavy meromyosin was much smaller than that of actin, showing that the rigor complex is formed in a first order reaction under these conditions. That is, the rate of the complex formation (k_c) was determined by the following equation:

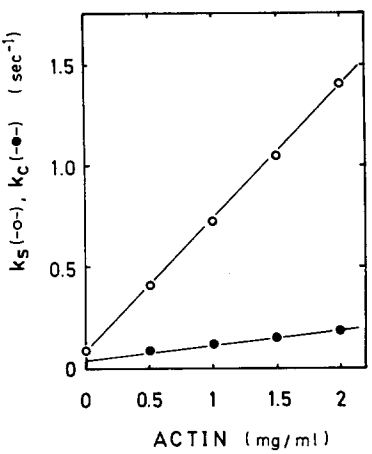
$$[(\Delta(\infty)-\Delta(t))/\Delta(\infty)] = \exp(-k_c t) \tag{1}$$

or

$$\ln[(\Delta(\infty)-\Delta(t))/\Delta(\infty)] = -k_c t \tag{1'}$$

where t stands for the time after the complex begins to form, and $\Delta(t)$ and $\Delta(\infty)$ are the intensities of the light-scattering at t and $t = \infty$.

We further investigated, simultaneously, the effect of actin concentration both on the rate of the steady Mg–ATPase activity of heavy meromyosin and on the rate of the retarded rigor complex formation in the same system, at low ionic strengths. The latter rate did not depend appreciably upon the amount of ATP present initially, indicating that the rate is independent of the concentration of ADP and P_i. Fig. 3 shows the results. The rate of the rigor complex formation was accelerated by actin as was the steady Mg–ATPase activity of heavy meromyosin. However, the extent of the acceleration of the former rate was far less than that of the latter. The rate of



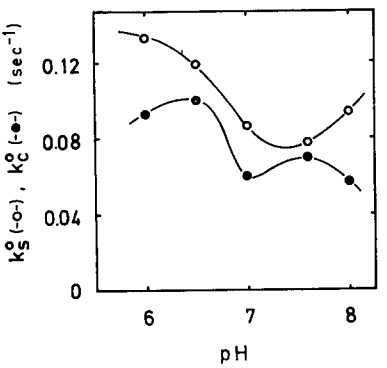
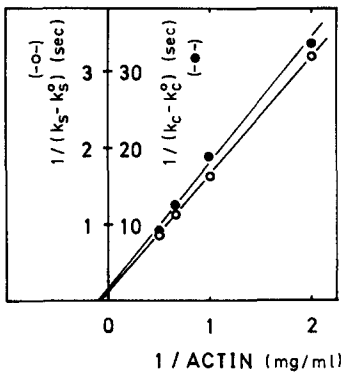


Fig. 3. Dependence on actin concentration of the rate of acto-heavy meromyosin ATPase, k_s (○–○), and the rate of rigor complex formation, k_c (●–●). 0.2 mg/ml heavy meromyosin, 0.1 M KCl, 10 mM Tris–maleate (pH 7), 1 mM $MgCl_2$ and 0.12 mM ATP at 20 °C.

Fig. 4. Dependence on pH of rate of heavy meromyosin Mg–ATPase, i.e. k_s^0 (○–○), and the rate of decomposition of myosin*–ADP–P_i, i.e. k_c^0 (●–●), in the absence of actin. 0.2 mg/ml heavy meromyosin, 0.1 M KCl, 1 mM $MgCl_2$ and 0.06 mM ATP at 20 °C. 20 mM buffers: Tris–HCl (pH 8 and pH 7.6) Tris–maleate (pH 7) and maleate buffer (pH 6.5 and pH 6).

the rigor complex formation extrapolated to zero actin concentration (k_c^0), which was expected to give that of the spontaneous decomposition of heavy meromyosin*–ADP–P_i, was about half that of the steady ATPase activity of heavy meromyosin in the absence of actin (k_s^0). It had been shown by the ultraviolet spectral change and the calorimetric studies, that the rate of the spontaneous decomposition of heavy meromyosin*–ADP–P_i occurring in a first order reaction after the end of ATP consumption, was about half of that of the steady Mg–ATPase activity of heavy meromyosin in the absence of actin [32]. Therefore, the rate (k_c^0) determined by our method gives that of the spontaneous decomposition of heavy meromyosin*–ADP–P_i, as expected. Hence Fig. 3, showing that the rigor complex formation is accelerated by actin, indicates that actin accelerates the decomposition of heavy meromyosin*–ADP–P_i. Fig. 4 shows the pH-dependencies of the spontaneous decomposition rate (k_c^0) and the steady Mg–ATPase rate (k_s^0). The pH-dependencies of these rates are different. The pH-dependency of the spontaneous decomposition rate is very similar to that obtained by Taylor et al. [29] (see the experimental points in their Fig. 7).

It has been well established that the acto-heavy meromyosin ATPase activity obeys the double-reciprocal plots of the excess ATPase rate ($k_s - k_s^0$) versus actin concentration [13, 33, 34]. We found that the actin-accelerated rigor complex formation obeys the same plots (Figs 5 and 6). The process of the actin-accelerated rigor complex formation was compared with the steady ATPase process by analyzing these double-reciprocal plots. The intercept of the abscissa represents the apparent binding constant (K_a) between actin and heavy meromyosin, and the intercept on the ordinate yields the maximum rate (V) in each process. The results obtained from Figs 5 and 6 are summarized in Table II. As the ionic strength increases, V in the complex formation



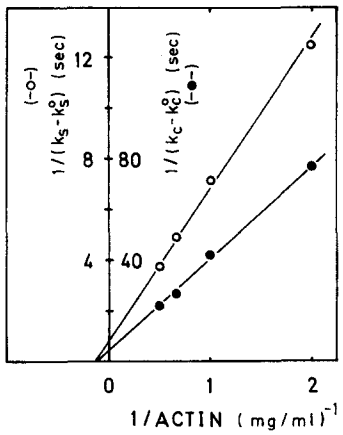


Fig. 5. Double-reciprocal plots of excess rates of acto–heavy meromyosin ATPase (○–○) and actin-accelerated rigor complex formation (●–●) versus actin concentration at 0.1 M KCl and 20 °C. 0.2 mg/ml heavy meromyosin, 10 mM Tris–maleate (pH 7), 1 mM $MgCl_2$ and 0.12 mM ATP. The excess rate in each process is defined as the observed rate minus the rate found in the absence of actin.

Fig. 6. Double-reciprocal plots of excess rates of acto–heavy meromyosin ATPase (○–○) and actin-accelerated rigor complex formation (●–●) versus actin concentration at 0.2 M KCl. Other experimental conditions are the same in Fig. 5.

process decreases with that in the steady ATPase process [34]. The pH-dependencies of V and K_a are shown in Fig. 7. Although the apparent binding constants in the two processes are nearly the same, the V of the actin-accelerated complex formation process is about 1/5 that of the steady ATPase process at each ionic strength and at each pH.

The good fit of these two processes to the plots means that an equilibrium exists in the binding between actin and heavy meromyosin, and the equilibrium complex formed between these is the rate-limiting intermediate in each process. The above four kinds of experiment suggest that the intermediate complex in the rigor complex formation is actin–myosin*–ADP–P_i. The retarded rigor complex formation such as is shown by Curve (a) in Fig. 1, itself indicates that the rate-limiting intermediate

TABLE II

V AND K_a IN STEADY ACTO–HEAVY MEROMYOSIN ATPase AND ACTIN-ACCELERATED RIGOR COMPLEX FORMATION AT TWO DIFFERENT KCl CONCENTRATIONS

Conditions were the same as in Figs 5 and 6.

	0.1 M KCl		0.2 M KCl	
	V (s^{-1})	K_a $(mg/ml)^{-1}$	V (s^{-1})	K_a $(mg/ml)^{-1}$
Steady state ATPase	7.1	0.09	1.02	0.17
Actin-accelerated rigor complex formation	1.1	0.08	0.26	0.15

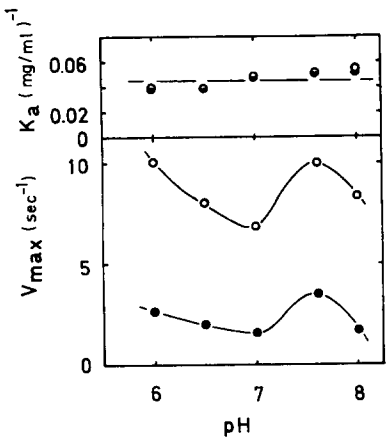


Fig. 7. Dependence on pH of V and K_a in steady acto-heavy meromyosin ATPase (○–○) and actin accelerated rigor complex formation (●–●). Conditions were the same in Fig. 4.

complex, i.e. actin–myosin*–ADP–P_i, scarcely contributes to the light scattering. This is probably because of its small concentration. Table II shows that actin and myosin*–ADP–P_i are almost completely dissociated to the same extent as are actin and heavy meromyosin in a Mg–ATP solution. Since the V in the ATPase process is larger than that in the rigor complex formation, the intermediate in the former process is not actin–myosin*ADP–P_i. In other words, the acto-heavy meromyosin ATPase activity cannot be explained merely by the actin-accelerated decomposition of myosin*–ADP–P_i. This means that the single route mechanism for the acto-heavy meromyosin ATPase proposed by Lymn and Taylor [12], in which actin–myo-

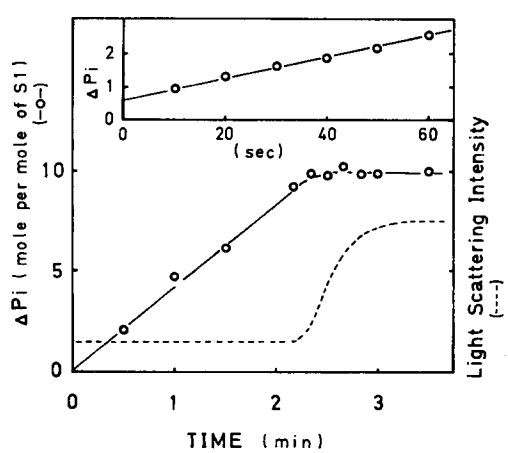


Fig. 8. Correlation of phosphate liberation (○–○) and time course of increase in light-scattering intensity (- - -) after adding subfragment 1 (S1) to a mixture of ATP and actin. 0.2 mg/ml subfragment 1, 1 mg/ml actin, 0.2 M KCl, 10 mM Tris–maleate (pH 7), 1 mM $MgCl_2$ and 0.02 mM ATP at 20 °C. The insert in the figure shows the subfragment 1-ATPase activity. 2 mg/ml subfragment 1, 0.2 M KCl, 10 mM Tris–maleate (pH 7), 1 mM $MgCl_2$ and 0.5 mM ATP at 20 °C. Note that the size of the pre-steady state burst of P_i liberation is 0.5 mole per mole of subfragment 1.

TABLE III

V AND K_a IN STEADY ACTO-SUBFRAGMENT 1 ATPase AND ACTIN-ACCELERATED RIGOR COMPLEX FORMATION

Conditions: 0.2 mg/ml subfragment 1, 0.2 M KCl, 10 mM Tris–maleate (pH 7), 1 mM $MgCl_2$ and 0.04 mM ATP at 20 °C.

	V (s^{-1})	K_a $(mg/ml)^{-1}$
Steady state ATPase	0.68	0.04
Actin-accelerated rigor complex formation	0.33	0.04

sin*–ADP–P_i is presumed to be the rate-limiting intermediate of the ATPase activity, is insufficient.

We obtained similar results in the acto–subfragment 1 system. Fig. 8 shows the time courses of the ATPase activity and the rigor complex formation in the system. The size of the pre-steady state burst of P_i liberation was about 0.5 mole per mole of subfragment 1 (see the insert of Fig. 8) [35]. The rigor complex of acto–subfragment 1 began to form slightly before the completion of ATP hydrolysis, in contrast to the acto–heavy meromyosin system, and about half of the maximum rigor complex formed at the end of ATP hydrolysis. In the acto-subfragment 1 system the two processes, i.e. the steady ATPase activity and the retarded rigor complex formation, could also be fitted by linear lines in the double-reciprocal plots. Table III shows the values of V and K_a. In this system the V of the steady ATPase process was twice as large as that of the retarded rigor complex formation, whereas the values of K_a were identical in the two processes.

DISCUSSION

In this paper we have shown that myosin*–ADP–P_i and actin are almost completely dissociated even in the absence of ATP, whereas heavy meromyosin and actin, mixed in an Mg–ADP solution, immediately form the rigor complex. Therefore, the kinetic property of heavy meromyosin–ADP–P_i; i.e. the heavy meromyosin–product (one to one) complex formed via ATP hydrolysis, is different from that of the heavy meromyosin–product complex formed by simply being mixed with ADP [4–8].

In our experiments it is shown that the size of the pre-steady state burst of P_i liberation from heavy meromyosin or subfragment 1 was one mole per two moles of the active site. The result is consistent with Tonomura [3], but not with Lymn and Taylor [36], who have reported a burst of one mole of P_i per mole of the active site. The contradiction between their results might be explained on the basis of an assumption in the former case, that heavy meromyosin molecules are a mixture of two kinds of heavy meromyosin i.e. one capable of producing a burst of two moles of P_i per mole of the enzyme and the other not capable of doing it. This is not correct, since, if it were, about half of the maximum amount of the rigor complex should form at the end of the complete ATP consumption.

A plausible explanation of our results

We are going to explain our results in terms of the assumption that the two

active sites of myosin are not identical. Evidence for this difference is: (1) The size of the burst of P_i is one mole per mole of heavy meromyosin or myosin, or one mole per two moles of myosin subfragment 1 (Figs 1, 2 and 3, see also ref. 3). (2) Incorporation of one mole of *p*-nitrothiophenol into one mole of myosin by a covalent bond results in the inactivation of one active site of myosin so that the P_i burst size becomes zero. *p*-Nitrothiophenyl-myosin hydrolyzes ATP at the other unmodified site and in this ATPase activity the rate-limiting intermediate is myosin–ATP [3], and the ATPase activity is initiated by actin (Table I; Takehara, S. and Tawada, K., submitted for publication). (3) One out of the two active sites of myosin is responsible for its ultraviolet absorbance spectrum change induced by the binding of ADP or ATP [37, 38]. In other words, we presume that one site of myosin forms myosin*–ADP–P_i as a rate-limiting intermediate while the other site forms myosin–ATP. Since the former site has a stronger affinity to ATP than the latter [3], the rigor complex formation of actin and heavy meromyosin sets in after the ATP consumption is almost completed. In the acto–subfragment 1 system, however, about half of the maximum amount of the rigor complex formed at the end of ATP consumption (compare Fig. 1 with Fig. 8). This can be understood by considering the difference of the two active sites. Half of subfragment 1 has a stronger affinity to ATP and forms the myosin*–ADP–P_i complex, whereas the other half forms the myosin–ATP complex. After the latter part of subfragment 1 completes formation of the rigor complex at the end of ATP consumption, as nitrothiophenylated heavy meromyosin shows, the formation of the rigor complex by the former subfragment 1 sets in.

We have shown that the rate-limiting intermediate in the retarded rigor complex formation of acto–heavy meromyosin is actin–myosin*–ATP–P_i. Hence, we may express the process of the rigor complex formation by the following scheme:

$$\begin{aligned}
&\text{A}+\text{myosin*–ADP–P}_\text{i} \underset{k_-^c}{\overset{k_+^c}{\rightleftharpoons}} \text{A–myosin*–ADP–P}_\text{i} \xrightarrow{k_0} \text{A}+\text{myosin}+\text{ADP}+\text{P}_\text{i} \\
&\text{myosin*–ADP–P}_\text{i} \xrightarrow{k_c^0} \text{myosin}+\text{ADP}+\text{P}_\text{i} \qquad (1) \\
&\text{A}+\text{myosin} \xrightarrow{\text{fast}} \text{A–myosin}
\end{aligned}$$

where A–myosin is the rigor complex. Under the condition that the total actin concentration is larger than the total heavy meromyosin concentration, we can obtain the following equation from the above scheme:

$$[\text{A–myosin}] = [\text{A–myosin}]_\infty[1-\exp(-k_c t)] \qquad (2)$$

where

$$k_c = k_c^0+(k_0-k_c^0)[\text{A}]/([\text{A}]+1/K_c) \qquad (3)$$

$$K_c = k_+^c/(k_-^c+k_0) \qquad (4)$$

$[\text{A–myosin}]_\infty$ = the total heavy meromyosin concentration.

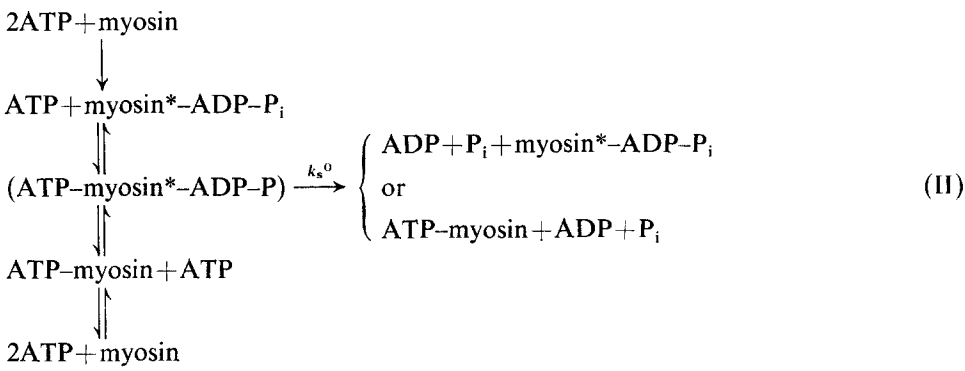
Eqn. 2 is identical to eqn. 1, since the rate-limiting intermediate complex, i.e. actin–myosin*–ADP–P_i, scarcely contributes to the light scattering because of its small concentration, and the increase in the light-scattering intensity is proportional to the

concentration of the rigor complex as pointed out already in Results. Re-writing eqn. 3, we obtain the double-reciprocal equation:

$$1/(k_c - k_c^0) = [1+(1/K_c)(1/[A])]/(k_0 - k_c^0) \quad (5)$$

From the comparison of eqn. 5 with Figs 5 and 6, it is apparent that $k_0 - k_c^0$ and K_c correspond to the V and K_a in the rigor complex formation.

We have shown that the rate-limiting intermediate in the actomyosin ATPase is not actin–myosin*–ADP–P_i: the V of the actomyosin ATPase cannot result merely from the actin-accelerated decomposition of myosin*–ADP–P_i. This could be explained by Tonomura's scheme (see ref. 39) since his scheme involves another process apart from the decomposition, i.e. Process (1) shown earlier in this paper. However, no explanation can be given from the scheme for the fact that the ATP hydrolysis by nitrothiophenylated heavy meromyosin, the rate of which is limited by bond hydrolysis, is accelerated by actin (see Table I). Hence, we propose an alternative scheme for the actomyosin ATPase. In the absence of actin, myosin hydrolyses ATP at the two non-identical active sites as follows:



(II)

Since both of the active sites of myosin have a strong affinity to ATP, we may consider that myosin stays predominantly as ATP–myosin*–ADP–P_i in the presence of sufficient ATP. The two types of ATP hydrolysis occurring at the two non-identical active sites are accelerated by actin as follows;

$$A+(\text{ATP–myosin*–ADP–}P_i) \underset{k_-{}^s}{\overset{k_+{}^s}{\rightleftharpoons}} A\text{–}(\text{ATP–myosin*–ADP–}P_i)$$

$$\xrightarrow{k_1} \begin{cases} A+ADP+P_i+\text{myosin*–ADP–}P_i & \text{(i)} \\ A+\text{ATP–myosin}+ADP+P_i & \text{(ii)} \\ A\text{–myosin}+2ADP+2P_i & \text{(iii)} \end{cases} \quad \text{(III)}$$

It is apparent that the above Scheme III obeys the double-reciprocal plots. In the above Scheme III, the Process iii might not be dominant since this process must accompany simultaneously the ATP hydrolysis at one site of heavy meromyosin and the decomposition of myosin*–ADP–P_i generated at another site. If this is not so, the cycles in the actomyosin ATPase scarcely involve the step of rigor complex formation. Our experiments do not distinguish which of the other two processes,

i or ii, is dominant. The above scheme for the actomyosin ATPase has been used in a model of muscle activity [40].

Table II and Fig. 7 indicate that the rate constant in the actin-accelerated rigor complex formation (k_c) is about five times smaller than that in the steady actomyosin ATPase (k_s), since k_s^0 and k_c^0 are small compared with V. Moreover, the apparent binding constant between myosin*–ADP–P_i and actin is identical to that between ATP–myosin*–ADP–P_i and actin. The acto–subfragment 1 ATPase activity obeys the double-reciprocal plots, although two kinds of intermediate complex, subfragment 1–ATP and subfragment 1*–ADP–P_i, are present simultaneously as shown above. This is because the apparent binding constant of subfragment 1–ATP to actin is about the same as that of subfragment 1*–ADP–P_i to actin as shown in Table III. The V in the acto–subfragment 1 ATPase is twice as large as that of the actin-accelerated rigor complex formation. This means that the rate of ATP hydrolysis with actin–subfragment 1–ATP as a rate-limiting intermediate is about the same as that of the actin-accelerated decomposition of subfragment 1*–ADP–P_i.

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