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TEMPERATURE-DEPENDENT TRANSITIONS OF THE MYOSIN-PRODUCT INTERMEDIATE AT 10 °C IN THE Mn(II)–ATP HYDROLYSIS

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SUMMARY

1. While below 10 °C, the initial burst of P_i liberation in the hydrolysis of Mn(II)–ATP by heavy meromyosin or myosin subfragment 1 was inhibited by the pre-addition of ADP without any change in the steady-state activity, it was not inhibited above 10 °C. The burst size was about one mole per two moles of myosin active sites.

2. Above 10 °C, the ultraviolet absorption spectrum of heavy meromyosin induced by ATP in $MnCl_2$ was similar to that induced in $MgCl_2$ and the spectral decay to the ADP-induced level occurred only after all the ATP in the solution was depleted. In contrast, below 10 °C the spectrum induced by ATP in $MnCl_2$ decayed to the ADP-induced level within a few seconds after the addition of ATP, although ATP was present in the solution.

3. These two results indicate that in Mn–ATP above 10 °C at the burst site there is a myosin*–ADP–P_i complex generated by ATP hydrolysis while below 10 °C there is a myosin-product complex identical with the one generated by adding ADP (and P_i) to myosin.

4. At temperatures both above and below 10 °C, the Mn–ATP hydrolysis of heavy meromyosin was activated by actin and superprecipitation of actomyosin occurred. Characteristics of these phenomena showed a transition at around 10 °C.

INTRODUCTION

The myosin–product complex formed by the hydrolysis of ATP (this complex is written myosin*–ADP–P_i) is distinct from the myosin–product complex generated by adding ADP (and P_i) to myosin (this complex is written myosin–ADP). The distinction has been shown by several examples. The ultraviolet absorption spectrum [1], electron spin resonance spectrum [2] and fluorescence emission spectrum [3] of myosin–ADP are different from those of myosin*–ADP–P_i. A calorimetric study on

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the degradation process of myosin*–ADP–P_i to myosin–ADP also suggested this difference [4]. The myosin*–ADP–P_i complex is a rate-limiting intermediate but not the myosin–ADP complex, in the Mg–ATP hydrolysis [5, 6]. This was shown by the fact that the initial burst of P_i liberation is not inhibited by adding ADP (and P_i) to a myosin solution prior to the addition of ATP [7].

It has been assumed that actomyosin ATPase is the actin-accelerated degradation of the myosin*–ADP–P_i intermediate; in addition a myosin cross-bridge in muscle develops tension when the myosin*–ADP–P_i intermediate on the cross-bridge is degraded by combining with actin [8–10]. It is important to test this assumption under various conditions.

Recently Yazawa et al. [11] reported the following observations in the presence of $MnCl_2$ at 8 °C: (1) an unusually rapid decay in the difference spectrum of heavy meromyosin induced by ATP and (2) complete inhibition of the burst of P_i liberation by the pre-addition of 2 moles of ADP per mole of heavy meromyosin, without any change in the steady ATPase rate. Their observations show that the myosin–ADP complex is a rate-limiting intermediate in the Mn–ATP hydrolysis at 8 °C, so that the initial burst of P_i liberation is inhibited by the pre-addition of ADP.

However, we found that at room temperature a burst of P_i liberation by heavy meromyosin in the Mn–ATP hydrolysis occurred irrespective of the pre-addition of ADP to the heavy meromyosin solution as in the Mg–ATP hydrolysis, and that at temperatures above 10 °C the myosin*–ADP–P_i complex was a rate-limiting intermediate in the Mn–ATP hydrolysis. At temperatures below 10 °C, the decomposition of myosin–ADP was a rate-limiting step as reported by Yazawa et al. [11]. Therefore, we studied in detail the effect of temperature on the ATPase activities of heavy meromyosin, acto–heavy meromyosin and the superprecipitation of actomyosin in the presence of $MnCl_2$.

MATERIALS AND METHODS

Actin was prepared by the method of Drabikowski and Gergely [12]. Myosin and heavy meromyosin were prepared from rabbit skeletal muscle by the method of Perry [13], and Lowey and Cohen [14], respectively. Myosin subfragment 1 was prepared by treating myosin with chymotrypsin according to the method of Onodera and Yagi [15]. Protein concentrations of actin and heavy meromyosin were determined by the biuret reaction. The concentration of myosin subfragment 1 was determined from absorbance at 280 nm and the extinction coefficient used was 770 cm^2/g for the protein [16].

The ATPase rate was determined by measuring the time course of P_i liberation. P_i was determined by the Martin and Doty procedure [17]. To start and stop the ATPase reaction within a few seconds, two syringes were used: one for injecting an ATP solution (0.5 ml) into a heavy meromyosin solution (1.5 ml) and another for injecting 2 ml of trichloroacetic acid (10 %) into the reaction mixture stirred by a magnetic stirrer. The syringe for injection of ATP had a water jacket for temperature control.

Viscosity measurements were made in an Ostwald-type viscometer. The experimental conditions are shown in the legend of Table I.

The difference spectrum of heavy meromyosin was measured by using a Carry

TABLE I

SPECIFIC VISCOSITY OF ACTIN, HEAVY MEROMYOSIN AND ACTO-HEAVY MEROMYOSIN IN A Mn–ATP SOLUTION CONTAINING ADP

Conditions: 0.15 M KCl, 20 mM Tris–maleate (pH 7.0), 2 mM $MnCl_2$, 200 μM ADP (initially), 1 mM ATP; 1 mg of actin per ml, 0.5 mg of heavy meromyosin per ml. Flow time of the solvent was 6.35 s at 20 °C and 9.65 s at 4 °C.

Temperature (°C)	Actin (a)	Heavy meromyosin (b)	a+b	Acto–heavy meromyosin	
				in ATP	no ATP (in ADP)
4	0.50	0.03	0.53	0.53	2.83
20	0.50	0.03	0.53	0.53	2.66

model 14 spectrophotometer, and the time course of the spectrum decay was measured by using a Shimazu model DOUBLE-40 DF spectrophotometer having a thermostated cell holder.

The extent of superprecipitation of actomyosin was followed spectrophotometrically by observing the change in turbidity at 660 nm. The rate of superprecipitation was defined by the inverse of the time necessary for the turbidity to reach the half maximum.

The molecular weights of heavy meromyosin and myosin subfragment 1 were taken to be 340 000 [18] and 100 000 [15], respectively.

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

RESULTS

P_i liberation by heavy meromyosin with Mn(II)–ATP as a substrate

In the presence of $MnCl_2$ and at low temperature heavy meromyosin showed an initial P_i burst (the burst size was about one mole per mole of the enzyme) which was, unlike that in $MgCl_2$, completely inhibited by the pre-addition of ADP (or ADP and P_i) without any change in the steady state ATPase activity, as had been shown by Yazawa et al. [11]. An example of the experiments at 4 °C is shown in Fig. 1a. At room temperature, however, heavy meromyosin showed the P_i burst irrespective of the pre-addition of ADP and the burst size was about one mole per mole of heavy meromyosin. An example of the experiments at 15 °C is shown in Fig. 1b. The steady Mn–ATPase activity was about ten times larger than that of Mg–ATPase.

We examined in detail the temperature dependence of the burst size obtained when three moles of ADP per mole of heavy meromyosin were pre-added (Fig. 2). The burst size transition from one mole to zero mole per mole of heavy meromyosin occurred at about 10 °C. The transition occurred in a narrow temperature range smaller than 1 °C. The temperature dependence of the burst size in 0.1 M KCl was the same in 0.2 M KCl. The temperature dependence of the steady ATPase rate is also shown in Fig. 2 and this was different above and below 10 °C. This is more easily seen in an Arrhenius plot of the steady state activity (Fig. 3). The plot showed a break

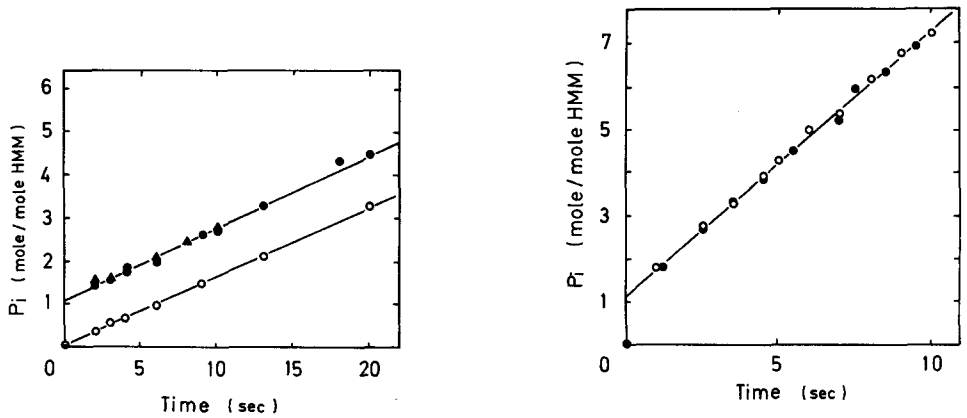

Fig. 1. Time courses of P_i liberation after the addition of ATP to heavy meromyosin (HMM) at 4 °C (a) and 15 °C (b). Conditions: 2.25 mg of heavy meromyosin per ml, 0.2 M KCl, 20 mM Tris–maleate (pH 7.0), 1 mM $MnCl_2$, 0.5 mM ATP and initial ADP concentration = 4× heavy meromyosin molarity (○) or 0 (●, ▲).

at about 10 °C and the slope below 10 °C was steeper than above 10 °C, unlike in the Mg–ATP hydrolysis. (An Arrhenius plot of Mg–ATPase gave a linear line over the entire corresponding temperature range [19].) Below 10 °C, the Arrhenius activation energy was about 12 kcal/mole, which was comparable to the value obtained in the Mg–ATP hydrolysis [19].

P_i liberation by myosin subfragment 1 with Mn–ATP as a substrate

The same analyses were carried out on myosin subfragment 1 and similar results were obtained. Above 10 °C the P_i burst was not inhibited by the pre-addition of ADP and the burst size was about 0.5 mole per mole of myosin subfragment 1. Since a heavy meromyosin molecule is composed of two "heads" [18], i.e. two sub-

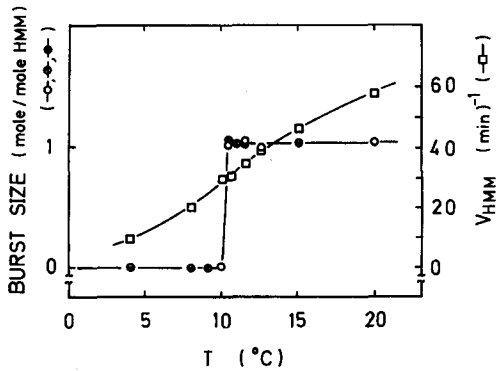

Fig. 2. Temperature dependences of the burst size in the presence of ADP (○, ⊙, ●) and steady ATPase rate (□) of heavy meromyosin (HMM). Conditions: 2 or 2.25 mg of heavy meromyosin per ml, 20 mM Tris–maleate (pH 7.0) (○, ●, □) or 20 mM Tris–HCl (pH 7.6) (⊙), 1 mM $MnCl_2$, 0.5 mM ATP, the initial concentration of ADP = 3× heavy meromyosin molarity, 0.2 M KCl (○, ⊙) or 0.1 M KCl (●, □).

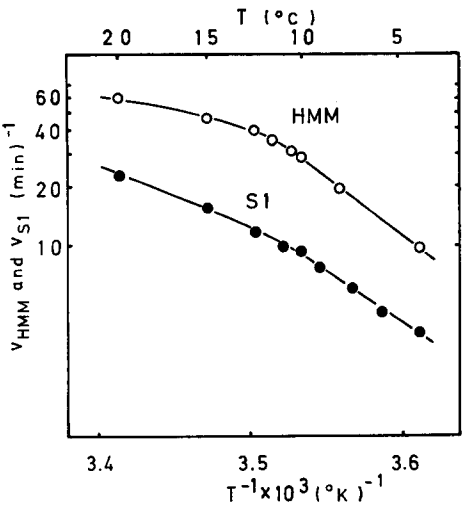

Fig. 3. Arrhenius plots of steady ATPase rates of heavy meromyosin (HMM) (○) and myosin subfragment 1 (S1) (●). Conditions: 0.5 mg of protein per ml, 0.1 M KCl, 20 mM Tris-maleate (pH 7.0), 1 mM $MnCl_2$, 0.5 mM ATP, initial concentration of ADP = 3× heavy meromyosin molarity.

fragment 1 molecules, this burst size coincides with that obtained with heavy meromyosin. Below 10 °C, on the other hand, the P_i burst was completely inhibited by the pre-addition of ADP (Fig. 4). An Arrhenius plot of the steady state ATPase activity of myosin subfragment 1 showed a break at about 10 °C (Fig. 3). Below 10 °C the slope was steeper than above 10 °C. Below 10 °C the activation energy was 12 kcal/mole. Thus, all the characteristics obtained with heavy meromyosin were preserved when the two heads of myosin were separated. This means that no interaction between the two heads of a heavy meromyosin is involved in the mechanism of either the P_i

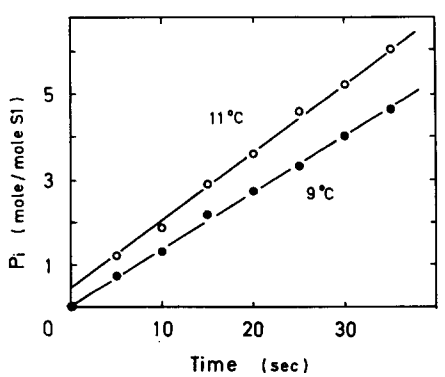

Fig. 4. Time courses of P_i liberation by myosin subfragment 1 after the addition of ATP at 9 °C (●) and at 11 °C (○) in the presence of ADP at a concentration equal to 4 times the molarity of subfragment 1. Conditions: 0.6 mg of myosin subfragment 1 per ml, 0.1 M KCl, 20 mM Tris-maleate (pH 7.0), 1 mM $MnCl_2$, 0.5 mM ATP.

burst or the steady state Mn–ATP hydrolysis, as in the case of Mg–ATP hydrolysis [20, 21].

Ultraviolet absorption difference spectrum of heavy meromyosin induced by ATP in $MnCl_2$

The fact that the P_i burst in the heavy meromyosin catalyzed Mn–ATP hydrolysis was inhibited by the pre-addition of ADP below but not above 10 °C means that the myosin*–ADP–P_i complex is a rate-limiting intermediate in the Mn–ATP hydrolysis above 10 °C whereas it is the myosin–ADP complex below 10 °C. In other words, the predominant steady-state complex generated at the active site responsible for the P_i burst (referred to as the burst site) is the myosin*–ADP–P_i complex above 10 °C while below 10 °C it is the myosin–ADP complex.

Yazawa et al. [11] had reported that the ultraviolet spectrum of heavy meromyosin induced by the addition of ATP in $MnCl_2$ rapidly decayed to the level of that induced by ADP at 8 °C and the decay rate was nearly constant (approx. 1 s^{-1}) regardless of the initial concentration of ATP. In contrast, we found that the difference spectrum induced by ATP in $MnCl_2$ at room temperature (e.g. 20 °C) did not begin to decay until the ATP was almost completely hydrolyzed. The final shape of the spectrum was identical to that induced by ADP (Figs 5 and 6). These difference spectra induced by ATP or ADP in $MnCl_2$ (Curve a and c in Fig. 5, respectively) and their molar extinction coefficients were very similar to the corresponding ones in $MgCl_2$ (Compare Fig. 5 with Fig. 1 in ref. 1.).

Time courses of the change in absorption at 289 nm are shown in Fig. 6. Patterns of the decay process were not the same at every temperature. At temperatures above 17 °C, the level of the absorption induced by ATP was preserved during the steady ATPase activity. At 15.2 °C and 12.6 °C, the absorption began to decay before the entire depletion of ATP in the solution. Still, the maximum level of the absorption induced by ATP was preserved for approximately 15 s at 12.6 °C, and then the ab-

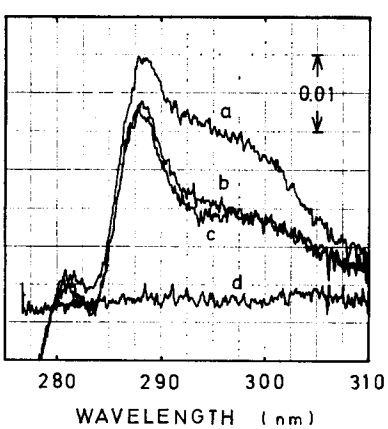

Fig. 5. Difference spectrum of heavy meromyosin induced by the addition of ATP at 20 °C. Conditions: 2.6 mg of heavy meromyosin per ml, 1 M KCl, 20 mM Tris–HCl (pH 8.0), 2 mM $MnCl_2$, 144 μM ATP. The times after adding ATP, when the scan passed through a wavelength of 290 nm, were 60, 490 and 760 s for curves a, b and c, respectively. Curve d is a trace of the base line. Ordinate: difference absorbance.

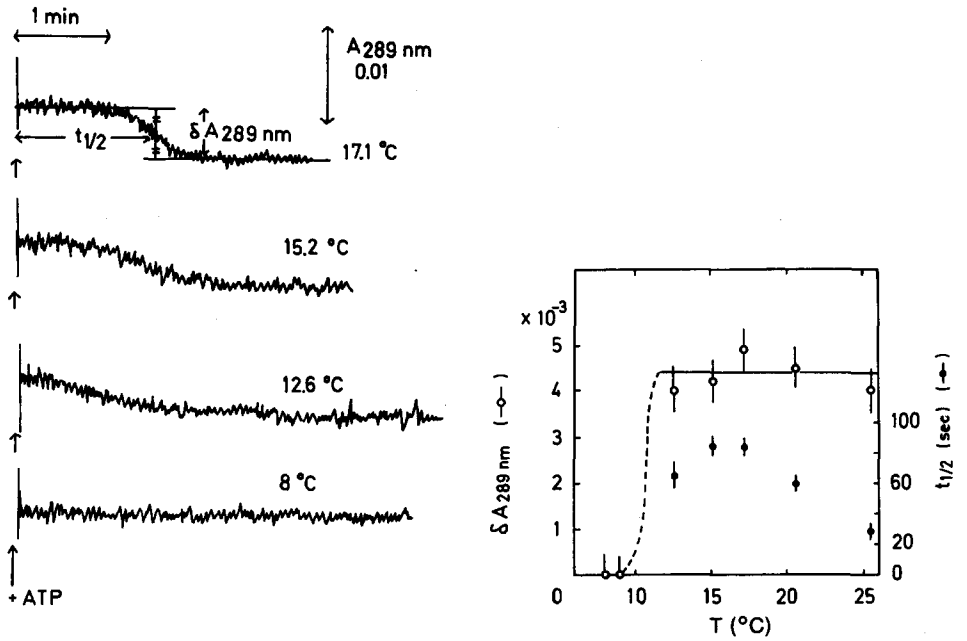

Fig. 6. Time course of the change in absorption at 289 nm of heavy meromyosin after adding ATP at various temperatures. Conditions: 2.5 mg of heavy meromyosin per ml, 0.2 M KCl, 20 mM Tris–HCl (pH 7.6), 1 mM $MnCl_2$, 270 μM ATP.

Fig. 7. Temperature dependence of the maximum extent of absorption change (○) and the half time (●). Conditions are the same in Fig. 6. The maximum extent of the absorption change ($\delta A_{289\ nm}$) was defined as the difference of the absorbance at about 0.1 min after adding ATP minus the absorbance after the complete change. The half time ($t_{\frac{1}{2}}$) was defined as the time when the change was half maximal.

sorption gradually decayed to the level induced by ADP as the ATP depletion proceeded. This indicates that the ATP-induced maximum level is preserved as far as the molar ratio of ADP to ATP does not exceed 1/4 at 12.6 °C, since the ATPase rate at 12.6 °C is approx. 10^{-2} μmoles/min/mg of heavy meromyosin. At 8 or 9 °C, in contrast, the level of the absorption even as early as 5 s after the addition of ATP was equal to that induced by ADP.

The "half time" ($t_{\frac{1}{2}}$) and the "maximum extent" of the change in the absorption at 289 nm at various temperatures, each defined in Fig. 6, are shown in Fig. 7. From the spectroscopic studies presented above, it is evident that there is a transition of the myosin–product intermediate at about 10 °C; above 10 °C the myosin–product intermediate is identical with the one generated by ATP hydrolysis when the ADP concentration is sufficiently small, while below 10 °C it is identical with the one generated by adding ADP to myosin.

The spectral decay occurring soon after ATP depletion in $MnCl_2$ at 20 °C was an exponential process, as in $MgCl_2$ [4], and the decay rate at 293 nm determined from a semilog plot of the absorbance versus time was about 0.02 s^{-1} in 1 M KCl (The plot is not shown here.). This value is comparable to that obtained in $MgCl_2$. However, the steady state ATPase rate in $MnCl_2$ was 0.2 s in 1 M KCl at 20 °C, that is about ten times larger than the spectral decay rate.

Viscosity of acto–heavy meromyosin solutions

Table I shows the results of viscosity measurements on acto–heavy meromyosin solutions. In a Mn–ATP solution containing ADP heavy meromyosin was almost completely dissociated from actin both above and below 10 °C, since the specific viscosity of the acto–heavy meromyosin solution was equal to the sum of the specific viscosities of actin and heavy meromyosin at 4 °C or 20 °C. The specific viscosity preserved the value of 0.53 until almost all ATP in the solution was hydrolyzed, at 4 °C or 20 °C. After the complete depletion of ATP, actin and heavy meromyosin bound firmly forming the rigor complexes. This means that they bind firmly in a Mn–ADP solution in which the myosin-ADP complex is formed.

Actin activation of heavy meromyosin Mn-ATP hydrolysis

It has been established that double-reciprocal plots of acto–heavy meromyosin Mg–ATPase $(v-v_0)$ versus actin concentration (A) are linear and can be expressed by the following empirical equation [22]:

$$1/(v-v_0) = [1+1/(K_aA)]/(V-v_0) \quad (1)$$

where v is the observed rate of ATP hydrolysis in the presence of actin; v_0 is the hydrolysis rate in the absence of actin and K_a is the apparent association constant.

In order to analyze the effect of temperature on the acto–heavy meromyosin ATPase in $MnCl_2$, we measured the ATPase rate as a function of actin concentration at various temperatures, and found that the double-reciprocal plots of the Mn–ATPase were also linear at every temperature. For the procedure to obtain the limiting rate (V) and the apparent association constant (K_a) from the double-reciprocal plot, see refs 15, 21, 22 and 25. The limiting rate and the apparent association constant obtained are shown in Fig. 8 as a function of the reciprocal of temperature.

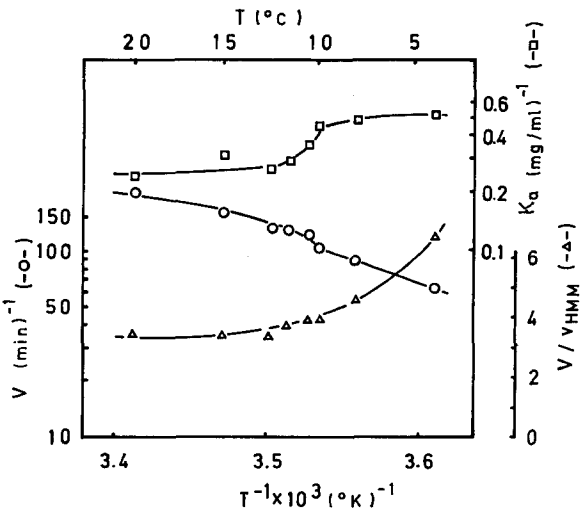

Fig. 8. Arrhenius plots of V (○) and K_a (□). The conditions are the same as in Fig. 3. (△) shows the extent of the actin activation of the Mn(II)–ATP hydrolysis activity of heavy meromyosin (HMM). $V_{HMM} = v_0$ in equation 1.

Note that the ordinate of the figure is expressed in a logarithmic scale and the limiting rate is shown in an Arrhenius plot. The units of V and K_a are (moles of ATP/mole of the enzyme/min) and (ml/mg of actin), respectively.

The curve of the apparent association constant versus T^{-1} showed a sigmoidal transition at about 10 °C. The apparent association constant below 10 °C was larger than above 10 °C. The curve of the limiting rate versus T^{-1} showed a break at about 10 °C. The slope in the Arrhenius plot of the limiting rate below 10 °C was slightly steeper than above 10 °C. Below 10 °C, the activation energy was 6 kcal/mole. This value was smaller than that for the acto-heavy meromyosin Mg-ATPase activity [19]. The limiting rate of the Mn-ATPase at room temperature was about 1/8 of that of Mg-ATPase [19], while the apparent association constant in Mn-ATPase was approximately the same in Mg-ATPase [19]. Fig. 8 shows that the extent of actin activation of the Mn-ATPase was largest at low temperature.

Effect of temperature on the superprecipitation of actomyosin in $MnCl_2$

At temperatures both below and above 10 °C, actomyosin showed superprecipitation in a Mn-ATP solution containing ADP. The rate and extent of superprecipitation were followed by measuring the increase in turbidity of the actomyosin solution at 660 nm. The extent of superprecipitation was nearly constant above 10 °C but showed a marked decrease below 10 °C (Fig. 9). This was in contrast to that in $MgCl_2$ which was greatest at low temperature [23].

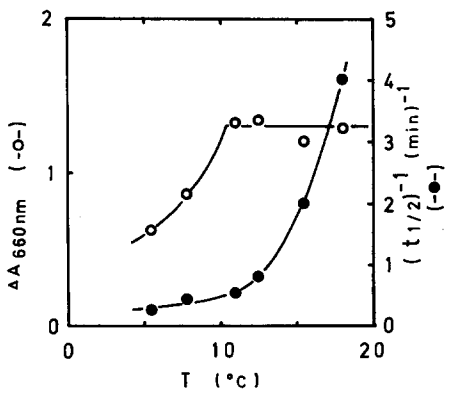

Fig. 9. Temperature dependences of extent (○) and rate (●) of superprecipitation of actomyosin. Conditions: 0.22 mg of actin per ml, 0.5 mg of myosin per ml, 50 mM KCl, 20 mM Tris-maleate (pH 7.0), 2 mM $MnCl_2$, 0.65 mM ATP, the initial concentration of ADP = 50 μM.

DISCUSSION

We showed in this paper that (i) above 10 °C the predominant steady-state intermediate generated at the burst site in a Mn-ATP solution was the myosin*-ADP-P_i complex while below 10 °C it was the myosin-ADP complex, (ii) the transition of the predominant steady-state intermediate from myosin*-ADP-P_i to myosin-ADP occurred critically at about 10 °C and (iii) all the characteristics of heavy meromyosin ATPase, acto-heavy meromyosin ATPase and the superprecipitation of actomyosin in Mn-ATP solutions varied in association with this transition. The

evidences for the conclusion (i) were that above 10 °C the P_i burst was not inhibited by the pre-addition of ADP but below 10 °C it was inhibited and that above 10 °C the ultraviolet absorption spectrum of heavy meromyosin in a Mn–ATP solution was identical with the one of the complex generated by ATP hydrolysis, while below 10 °C it was identical with that of the complex generated by adding ADP to myosin. Conclusions (ii) and (iii) were apparent from the results shown in Figs 2 and 7, and Figs 8 and 9, respectively.

The sharp transition of the intermediate mode from myosin*–ADP–P_i to myosin–ADP occurring in such a narrow temperature range (1 °C) cannot be understood merely by the difference in the temperature dependence of the affinities of the active site to ATP and ADP, because the temperature dependence takes an exponential form as $\exp(-\Delta H/RT)$. The following explanations are conceivable: transitional change of the conformation of myosin occurs in $MnCl_2$ at about 10 °C so that the temperature dependence of the affinity to ATP or ADP is different above and below 10 °C. If this were so, above 10 °C the active site might have a stronger affinity to ATP than to ADP while below 10 °C its affinity was stronger for ADP than for ATP. Our preliminary ESR study on a Mn–heavy meromyosin solution shows that a transitional change of the myosin conformation occurs at about 10 °C.

The burst size in the Mn–ATP hydrolysis was about one mole per mole of heavy meromyosin or per two moles of myosin subfragment 1, i.e. per two moles of myosin active sites. In the Mg–ATP hydrolysis by myosin, heavy meromyosin and myosin subfragment 1, the same burst size was obtained by us [25], and Tonomura and his colleagues [6, 20]. However, Lymn and Taylor [24] reported that the size was two moles per mole of myosin. The reason for this disagreement has not been clarified. It is not likely that our heavy meromyosin preparation is a mixture of two kinds of myosin: one capable of producing two moles of the P_i burst per mole of the enzyme and another that is not. If this were so, in a solution containing both Mn–ATP and Mn–ADP half of the heavy meromyosin would combine with actin below 10 °C, since heavy meromyosin in an ADP solution in which the myosin–ADP complex is formed, binds tightly to actin and the active site responsible for the P_i burst is masked with ADP in a solution containing both Mn–ATP and Mn–ADP below 10 °C. However, heavy meromyosin is almost completely dissociated from actin under these conditions as shown in Table I.

Our result that the burst size is one mole per mole of myosin does not necessarily reject the possibility that the two active sites of a myosin molecule are identical and both participate in the P_i burst reaction, because the figure could be reduced by impurities and inactive enzyme. However, neither the assumption of two identical active sites is consistent with the results shown in Table I, since this assumption implies that all the heavy meromyosin would combine with actin in a solution containing both Mn–ATP and Mn–ADP below 10 °C. Therefore, it is more likely that one of the two active sites (referred to as the burst site) binds the product and the other site (referred to as the second site) binds the substrate. Such a distinction between the two active sites has been reported [6, 25, 26]. Further stronger evidence for this distinction is that only one mole of the bound ATP out of the two moles induces the difference spectrum related to the reaction accompanying the P_i burst in $MnCl_2$ or $MgCl_2$ [11, 27]. In the following discussion, we assume the existence of two different active sites.

Our results may be expressed by the following scheme proposed by Yazawa et al. for the ATPase reaction [11]. It is assumed that one of the two different active sites of myosin relates to the ATPase reaction accompanying both the P_i burst and the spectral change (I) and the other to a simple ATPase reaction (II).

$$E(I)+ATP \overset{(a)}{\rightleftharpoons} E(I)^*\text{–}ATP \overset{(b)}{\rightleftharpoons} E(I)^*\text{–}ADP\text{–}P_i \overset{(c)}{\longrightarrow}$$

$$E(I)\text{–}ADP+P_i \overset{(d)}{\rightleftharpoons} E(I)+ADP+P_i,$$

$$E(II)+ATP \overset{(e)}{\rightleftharpoons} E(II)\text{–}ATP \overset{(f)}{\longrightarrow} E(II)\text{–}ADP+P_i \overset{(g)}{\rightleftharpoons}$$

$$E(II)+ADP+P_i,$$

where E represents myosin. In this scheme only the minimum, necessary number of intermediates are considered for the explanation of our results; $E(I)^*\text{–}ADP\text{–}P_i$ represents an intermediate containing trichloroacetic acid-labile P_i. The ATP-induced difference spectrum is mainly due to $E(I)^*\text{–}ADP\text{–}P_i$ [11]. E(I)–ADP represents a complex of the enzyme and ADP responsible for the ADP-induced difference spectrum [11]. In $MnCl_2$, above 10 °C the steps (c) and (f) limit the rate in reaction (I) and (II), respectively, while below 10 °C the steps (d) and (f) limit the rate in reaction (I) and (II), respectively.

At 20 °C the degradation rate of the myosin*–ADP–P_i intermediate to the myosin–ADP complex was 0.02 s^{-1} which is only 10 % of the steady Mn–ATPase rate. This means that the ATPase rate at the burst site (the reaction (I)) is very slow and that the second site predominates in the Mn–ATP hydrolysis at 20 °C, although this is not conclusive because we lack knowledge about the equilibrium in this step (b). The same argument has been used to determine the rate at each site in the heavy meromyosin Mg–ATP hydrolysis [26].

It is not apparent from our experiments whether or not actin accelerates both the degradation of myosin*–ADP–P_i occurring at the burst site and the ATP hydrolysis at the second site in $MnCl_2$ above 10 °C. We have shown elsewhere that actin accelerates both the degradation of the complex at the burst site and the ATP hydrolysis at the second site in Mg–ATP hydrolysis [25].

Below 10 °C the myosin–ADP complex is formed at the burst site; yet the Mn–ATP hydrolysis is activated by actin. The actin activation of the ATP hydrolysis below 10 °C is probably due to either actin-accelerated dissociation of the products from the myosin–ADP complex or actin-activated bond hydrolysis of ATP at the second site, or due to both. This in any case is not compatible with the current views on the actomyosin ATPase; that is, the actin-accelerated degradation of the myosin*–ADP–P_i intermediate [9].

Fig. 8 in this paper showed that the apparent association constant in the acto–heavy meromyosin Mn–ATP hydrolysis became suddenly large as the temperature was lowered to below 10 °C. The simplest explanation for this larger value below 10 °C is that heavy meromyosin with the burst site occupied with ADP has a stronger affinity to actin than heavy meromyosin with the burst site forming $E(I)^*\text{–}ADP\text{–}P_i$ when the second site binds ATP.

Actomyosin superprecipitated in a Mn–ATP solution although the rate of superprecipitation was very small at low temperatures and its extent became smaller as the temperature was lowered below 10 °C. The occurrence of superprecipitation

below 10 °C indicates that the generation of the myosin*–ADP–P_i complex as a rate-limiting intermediate at the burst site is not necessary for superprecipitation. Previously it was shown that actomyosin composed of myosin, the burst site of which was inactivated with *p*-nitrothiophenol, could not superprecipitate [6] although it showed the actomyosin ATPase [25]; the burst site modified with *p*-nitrothiophenol could not bind ADP [Takehara, unpublished]. Therefore, it is suggested that either the generation of the myosin*–ADP–P_i complex or the myosin–ADP complex as the rate-limiting intermediate at the burst site is required for the superprecipitation.

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