Interaction of Actin with *N*-Ethylmaleimide Modified Heavy Meromyosin in the Presence and Absence of Adenosine Triphosphate†

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ABSTRACT: *N*-Ethylmaleimide modified heavy meromyosin is only 3-fold activated by actin rather than 200-fold as is normal heavy meromyosin (Silverman, R., Eisenberg, E., and Kielley, W. W. (1972), *Nature (London) 240,* 207). Ultracentrifuge studies demonstrated that in the absence of ATP the *N*-ethylmaleimide modified heavy meromyosin binds to actin at a ratio of 2 actins to 1 *N*-ethylmaleimide modified heavy meromyosin. However, it was found that most of the *N*-ethylmaleimide modified heavy meromyosin was not bound to actin during ATP hydrolysis. Ultracentrifuge studies demonstrated that in the presence of 25 or 50 m*M* KCl under conditions where the ATPase is maximally activated by actin, less than 5% of the *N*-ethylmaleimide modified heavy meromyosin was bound to actin. In the absence of KCl there was limited binding but even this binding did not appear to correlate with the *N*-ethylmaleimide modified heavy meromyosin ATPase rate. Turbidity and viscosity studies also indicated that in the presence of ATP under conditions of maximal actin activation the *N*-ethylmaleimide modified heavy meromyosin and actin are almost completely dissociated, whereas there is a marked increase in turbidity and viscosity after all of the ATP is hydrolyzed. These results suggest that in the presence of ATP and actin *N*-ethylmaleimide modified heavy meromyosin exists most of the time in a refractory state unable to bind to actin and only a small part of the time in a nonrefractory state which can interact with actin. It follows that the major rate-limiting step during actin activation is the transition from the refractory to the nonrefractory state. Since the actin activation of *N*-ethylmaleimide modified heavy meromyosin is lower than that of normal heavy meromyosin this transition may be slower for *N*-ethylmaleimide modified heavy meromyosin than for normal heavy meromyosin.

Muscle is a highly organized structure composed primarily of the proteins myosin and actin. These proteins are arranged in the muscle cell as partially overlapped arrays of myosin and actin filaments. As the muscle contracts there is a cyclic interaction between the actin filaments and the heads of the myosin molecule (Huxley, 1969). This interaction activates the hydrolysis of ATP which is bound to the head of the myosin molecule. The chemical energy derived from this reaction is transduced into mechanical energy causing the two filaments to slide past each other as the muscle shortens. In the absence of ATP X-ray studies suggest that almost all of the myosin heads interact with actin but in the presence of ATP even during contraction a large fraction of the bridges appear to be dissociated from the actin (Huxley and Brown, 1967; Miller and Tregear, 1970).

In vitro studies have also shown a similar phenomenon. In the absence of ATP each myosin head binds to an actin molecule (Eisenberg et al., 1972b) whereas in the presence of ATP under conditions where actin strongly activates the HMM ATPase[1] a large fraction of the HMM is dissociated from actin (Eisenberg et al., 1972a). Evidence for this was derived from binding experiments at 0° using a number of different techniques including direct observations in the analytical ultracentrifuge. This latter technique demonstrated that more than two-thirds of the HMM heads remained unbound to actin at high actin concentration where the HMM ATPase is close to its maximal value, i.e. V_{max}. On the basis of this finding Eisenberg and Kielley (1972) suggested that during the cyclic interaction of actin with HMM in the presence of ATP the HMM heads can exist in two states: a refractory state unable to bind to actin and a nonrefractory state which can bind to actin. Since a large part of the HMM is not bound to actin during the cycle it follows that the conversion from the refractory to the nonrefractory state must be the rate-limiting step in the cycle. After conversion from the refractory to the nonrefractory state HMM then binds to actin, and rapidly releases the products of ATP hydrolysis and then returns to the refractory state in the presence of ATP to complete another cycle. Lymn and Taylor (1970) demonstrated that at 0° there is an initial fast hydrolysis in the presence of actin which is more than 10 times faster than the steady-state rate. They demonstrated that actin combines with the myosin product complex and displaces the products of hydrolysis. Since the release of product in this model is rate limiting this would predict that all of the actin and myosin would be complexed at V_{max} unless there were a slow rate-limiting step after the initial fast hydrolysis of ATP. Since binding experiments demonstrated that at high actin concentration in the presence of ATP a large part of the HMM does not bind to actin, Eisenberg et al. (1972a) postulated that a slow step from the refractory to the nonrefractory state occurred following the initial rapid hydrolysis of ATP.

Studies on the kinetics of the myosin ATPase reaction have demonstrated that there are two sulfhydryl groups involved in the hydrolysis of ATP. Modification of one of these groups (SH_1) with the sulfhydryl reagent MalNEt[1] results in a fourfold increase in Ca^{2+} ATPase activity and a loss of K^+ ATPase activity, whereas modification of both

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[1] Abbreviations used are: HMM ATPase, heavy meromyosin adenosine triphosphatase; MalNEt, *n*-ethylmaleimide.

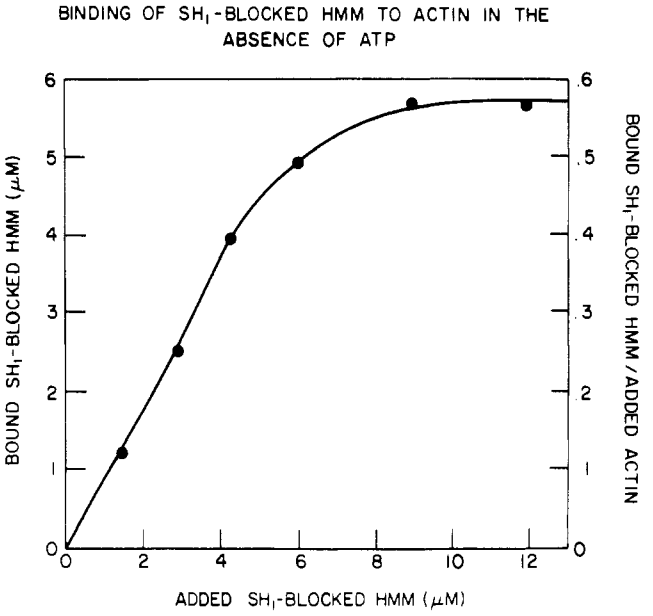


FIGURE 1: Binding of SH_1-blocked HMM to F-actin. The analytical ultracentrifuge was employed to measure the binding as described under Materials and Methods. Conditions: 0.1 *M* KCl, 1 m*M* $MgCl_2$, 10 m*M* imidazole (pH 7.0), 5 m*M* P_i, and 10 μM actin, T = 15°.

the SH_1 and SH_2 results in a loss of both Ca^{2+} and K^+ ATPase activity (Sekine and Kielley, 1964; Yamaguchi and Sekine, 1966). Recently, Silverman et al. (1972) found that actin activation of the SH_1 blocked HMM was greatly reduced as compared to actin activation of the normal HMM: only 3-fold actin activation occurs with SH_1-blocked HMM compared to 200-fold activation with normal HMM. The question therefore arises as to which step in the cycle of myosin–actin–ATP interaction is affected by MalNEt modification of the SH_1 group of myosin. If product release from the acto-HMM complex were slowed down in the cycle so that it became rate limiting then most of the SH_1-blocked HMM would be complexed with actin at high actin concentration where the ATPase is close to V_{max}. On the other hand, if the rate of conversion from the refractory to the nonrefractory state were reduced so that it was rate limiting most of the SH_1-blocked HMM would be unbound to actin at V_{max}. A reduction in either of these rates would explain the decrease in the actin activation observed with SH_1-blocked HMM. To determine which of the two rates was decreased during ATP hydrolysis under conditions of maximum actin activation, binding studies in the analytical ultracentrifuge as well as viscosity and turbidity studies were employed. The results indicated that as with normal HMM very little of the SH_1-blocked HMM is bound to actin during its cycle of interaction with actin and ATP suggesting that blocking SH_1 may have reduced the rate of conversion of HMM from the refractory to the nonrefractory state.

Materials and Methods

Rabbit skeletal myosin was prepared by the method of Kielley and Harrington (1960), and HMM was prepared from myosin by the method of Eisenberg and Moos (1968). Actin was prepared by a modified method of Spudich and Watt (1971) (Fraser et al., 1975). For binding experiments in the absence of ATP, the actin was mixed with a 50% suspension of Dowex 1-X anion exchange resin (0.1 vol of actin). The mixture was then filtered through a fritted glass Buchner funnel to remove the Dowex resin. The MalNEt

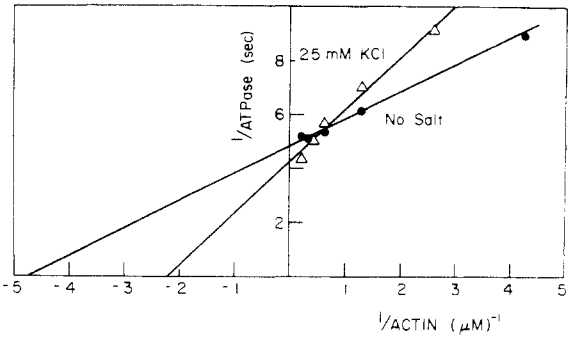


FIGURE 2: Double-reciprocal plots of acto-SH_1-blocked HMM-ATPase vs. added actin concentration: (●) no salt; (△) 25 m*M* KCl. 1/ATPase = μmol of SH_1-blocked HMM/sec per μmol of P_i. For each plot the rate of the SH_1-blocked HMM ATPase in the absence of actin was subtracted from the measured ATPase rate (SH_1-blocked HMM ATPase; no salt = 0.048 sec^{-1}, 25 m*M* KCl = 0.06 sec^{-1}).

used to modify myosin was resublimed before use. SH_1-blocked HMM was prepared from SH_1-blocked myosin by a modified method of Sekine and Kielley (1964). Myosin at 10 mg/ml was incubated with a fourfold excess of MalNEt per myosin subunit. Conditions for the reaction were 0.5 *M* KCl and 20 m*M* imidazole (pH 7.0), temperature 0°. After 40 min the reaction was stopped by the addition of a 25-fold excess of dithiothreitol. The protein was then concentrated with ammonium sulfate (50%). Protein concentrations were determined by ultraviolet (uv) absorption using the following extinction coefficients: 543 cm^2/g at λ 280 nm for myosin (Gellert and Englander; 1963); 647 cm^2/g at λ 280 nm for HMM (Young et al., 1964); and 1149 cm^2/g at λ 280 nm for F-actin (Eisenberg and Moos, 1967). ATPase rates were measured with a pH-Stat as previously described (Eisenberg and Moos, 1967). The titrating base used was 50 m*M* KOH delivered from a 0.25-ml syringe. All of the reactions were performed under these conditions unless otherwise stated: 3 m*M* $MgCl_2$, 3 m*M* ATP, 3 m*M* imidazole (pH 7.0), 2 m*M* P_i, 5.8 μM SH_1-blocked HMM. The temperature was 15°.

Viscosity measurements were performed in a 15° water bath with Ostwald viscometers having an average outflow time for water of 12 sec. Each reaction was initiated by the addition of SH_1-blocked HMM to the reaction mixture in a beaker. The reaction mixture was stirred for 30 sec at 15° before 6.0 ml was transferred to the viscometer.

Turbidity studies were performed at 350 nm in a Cary 14 spectrophotometer. The sample in the cuvet was maintained at 15° by means of a water jacket. Each sample was stirred in a beaker in a 15° water bath for 30 sec before it was transferred to the cuvet.

Ultracentrifuge experiments were performed at 15° in a Model E Analytical Ultracentrifuge equipped with a photoelectric scanner as described by Eisenberg et al. (1972a). Each sample was incubated in a 15° water bath and transferred into a 12-mm double-sector cell as soon as possible after the initiation of the reaction. The cells were then placed in a two-hole aluminum rotor which was also maintained at 15°. The sample was centrifuged at 30,000 rpm and scans were taken at 288 nm to minimize absorbance due to ATP.

Results

Eisenberg et al. (1972a) employed the analytical ultracentrifuge to determine the amount of HMM which was bound to actin both in the presence and absence of ATP.

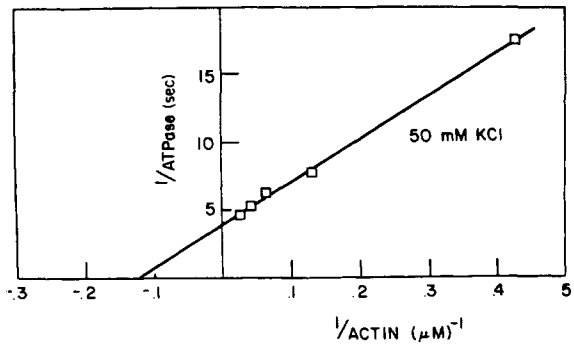


FIGURE 3: Double-reciprocal plot of acto-SH_1-blocked HMM-ATPase vs. added actin concentration in the presence of 50 mM KCl. The rate of SH_1-blocked HMM ATPase in the absence of actin was subtracted from the measured ATPase rate (SH_1-blocked HMM-ATPase = 0.059 sec^{-1}).

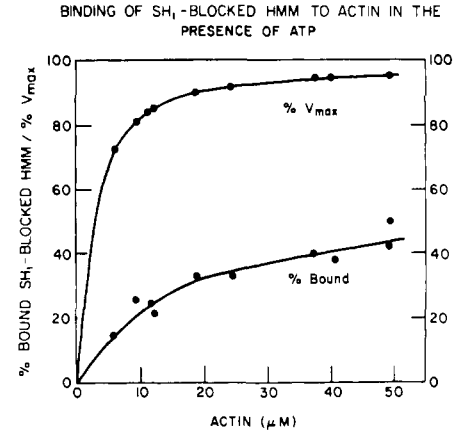


FIGURE 4: Binding of SH_1-blocked HMM to actin in the presence of ATP. The analytical ultracentrifuge was employed to measure the binding as described under Materials and Methods. The percent V_{max} was calculated from the double-reciprocal plot in the absence of salt (Figure 2).

Since actin but not HMM rapidly sediments under the conditions employed any HMM bound to actin will sediment with the actin and the rest will remain in the supernatant. Before looking at the binding of SH_1-blocked HMM to actin in the presence of ATP we investigated whether blocking SH_1 affects actin-HMM binding in the absence of ATP.

Ultracentrifuge experiments on normal HMM showed that in the absence of ATP HMM binds to actin in a ratio of 1 mol of HMM per 2 mol of actin (Eisenberg et al., 1972b). Figure 1 shows that as increasing amounts of SH_1-blocked HMM were added to 10 μM actin in the absence of nucleotide the binding was nearly stoichiometric and the actin was saturated at 2 mol of actin per mol of SH_1-blocked HMM, which indicates that blocking SH_1 had no apparent effect on the binding of HMM to actin in the absence of ATP.

Before a similar binding experiment could be performed in the analytical ultracentrifuge in the presence of ATP the actin concentration where the actin-activated ATPase is close to V_{max} had to be determined. Only under conditions of V_{max} where the binding to actin is not itself rate limiting can it be determined which other step in the cycle has been affected by MalNEt modification. Figure 2 shows a double-reciprocal plot of the SH_1-blocked HMM ATPase rate vs. actin concentration in the absence of KCl. From the intercept on the ordinate the V_{max} was determined to be 0.208 sec^{-1} which represents a threefold actin activation of the SH_1-blocked HMM ATPase. From the intercept on the abscissa the apparent binding constant of the actin to the SH_1-blocked HMM-ATP complex was calculated to be 2 μM so that at 45 μM actin the ATPase was 95% V_{max}. Figures 2 and 3 show similar reciprocal plots for experiments performed at 25 and 50 mM KCl, respectively. The increased salt concentration had very little effect on V_{max} but did decrease the apparent binding constant as is also true for normal acto-HMM. However, since the apparent binding constant of SH_1-blocked HMM-ATP to actin is considerably stronger than with normal HMM, even at 50 mM KCl, the ATPase is 86% V_{max} in the presence of 52 μM actin. Therefore, in contrast to the situation with normal HMM the ATPase can be brought close to V_{max} at 25 and 50 mM KCl and the analytical ultracentrifuge can be employed to determine the rate-limiting step in the cycle at these higher salt concentrations.

In measuring the binding of SH_1-blocked HMM to actin in the presence of ATP, the ATP concentration was chosen

Table I: Effect of KCl on the Binding of SH_1-Blocked HMM to Actin.[a]

Actin (μM)	[KCl] (nM)	% V_{max}	% Bound
45	None	95	40
45	25	91	4
52	50	86	0

[a] The % V_{max} was calculated from the appropriate plots on Figures 2 and 3. The binding studies were performed in the analytical ultracentrifuge as described under Materials and Methods.

so that the SH_1-blocked HMM not bound to actin during ATP hydrolysis could be observed by the photoelectric scanner before all of the ATP was hydrolyzed. The experiments were performed at 15° to allow time for the actin to sediment some distance away from the meniscus before all of the ATP was hydrolyzed. Figure 4 shows the results of the binding experiment in the absence of salt. In the absence of salt as with normal HMM at 0°, 40% of the SH_1-blocked HMM was bound to actin when the reaction was very near V_{max} but even this limited binding seemed to be unrelated to the ATPase activity (Table I). If the limited binding observed in the absence of salt was related to actin activation of the SH_1-blocked HMM ATPase then the binding should correlate with the ATPase rate. However, as shown in Figure 4, no correlation was observed. As the actin concentration was increased from 10 to 50 μM the binding doubled even though the ATPase rate was already nearly V_{max}. Therefore, it appears that even the limited binding observed in the absence of salt is unrelated to the actin-activated ATPase. Supporting this idea we found that in the presence of 25 and 50 mM KCl less than 5% of the SH_1-blocked HMM remains bound to actin when the ATPase is near V_{max} (Table I). Our results suggest that the rate-limiting step in the cycle occurs before the SH_1-blocked HMM binds to actin, i.e. in the conversion from the refractory to the nonrefractory state.

The control experiments indicated in Table II were performed to further investigate the binding of SH_1-blocked HMM to actin in the absence of salt. Lines 1 and 2 show that when the SH_1-blocked HMM concentration was halved then the amount of SH_1-blocked HMM remaining

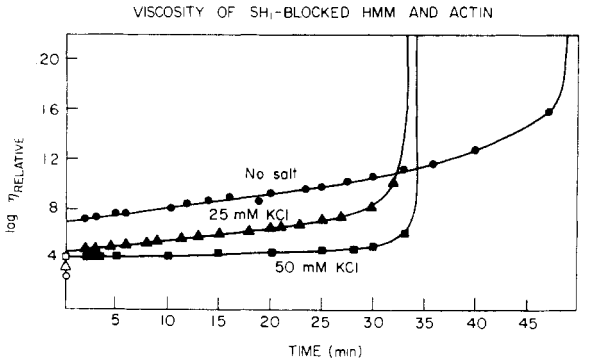


FIGURE 5: The viscosity of SH_1-blocked HMM and actin in the presence of ATP. The viscosity measurements were measured as described under Materials and Methods both in the absence and presence of 25 and 50 mM salt.

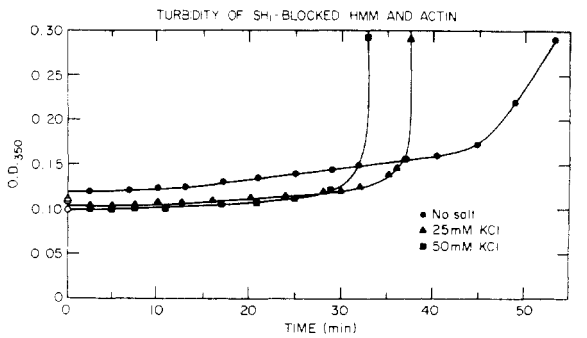


FIGURE 6: The turbidity of SH_1-blocked HMM and actin in the presence of ATP. The turbidity measurements were measured as described under Materials and Methods both in the absence and presence of 25 and 50 mM salt.

Table II: Binding of SH_1-Blocked HMM to Actin.[a]

Actin (μM)	(NalNEt–HMM) (μM)	Nucleotide	% V_{max}	% Bound
40	5.7	ATP	95	38
40	2.86	ATP	95	39
40	5.7	ADP		100

[a] Conditions were the same as described under Materials and Methods except that sample 3 was centrifuged after all of the ATP had been hydrolyzed to ADP + P_i. These experiments were performed in the absence of salt.

Table III: Comparison of Actin Activation of SH_1-Blocked Myosin and SH_1-Blocked HMM.[a]

Protein	ATPase (sec^{-1}) No Actin	22.2 μM Actin	45 μM Actin
SH_1-blocked myosin	0.45	1.27 (2.8-fold)	1.38 (3.1-fold)
SH_1-blocked HMM	0.42	1.16 (2.8-fold)	1.28 (3.1-fold)

[a] Conditions: 60 mM KCl, 2 mM ATP, 4 mM $MgCl_2$, 2 mM imidazole, pH 7.0, T = 25°.

in the supernatant was also halved. This indicates that the observed absorbance in the supernatant is indeed due to the presence of SH_1-blocked HMM rather than to unsedimented actin. After all of the ATP was hydrolyzed to ADP and P_i the SH_1-blocked HMM should all be bound to actin and line 3 shows that this was indeed the case.

Further evidence that most of the SH_1-blocked HMM is not bound to actin during ATP hydrolysis was obtained from viscosity and turbidity studies. These studies were performed under the same conditions as the ultracentrifuge experiments described above, i.e. at an actin concentration where the ATPase is very close to V_{max}. Figure 5 shows the results of the viscosity measurements at 0, 25, and 50 mM KCl. The open symbols on the graph indicate the sum of the log of the relative viscosities of the SH_1-blocked HMM and actin measured individually. This is the viscosity that would be expected if no interaction occurred. The solid symbols show the actual measured viscosity during ATP hydrolysis. In the presence of 25 and 50 mM KCl the theoretical and experimental values were essentially identical suggesting that most of the SH_1-blocked HMM is not bound to actin under these conditions. After all of the ATP was hydrolyzed there was a marked increase in viscosity as would be expected from the ultracentrifuge results which show that almost all of the SH_1-blocked HMM binds to actin after the ATP is hydrolyzed. In the absence of salt there was a small increase in the presence of ATP above the theoretical viscosity expected under these conditions but the increase was much less than occurred after all of the ATP was hydrolyzed. This suggests there is some interaction between SH_1-blocked HMM and actin in the absence of salt which agrees with the ultracentrifuge studies described above.

Turbidity studies also showed that there was very little interaction between SH_1-blocked HMM and actin during ATP hydrolysis under conditions of maximum actin activation. Figure 6 shows that either in the presence or absence of KCl there was virtually no increase in turbidity above that of the completely dissociated system, whereas there was a large increase in turbidity after all of the ATP was hydrolyzed. This is further evidence that during ATP hydrolysis almost all of the SH_1-blocked HMM is free of actin even under conditions of maximal actin activation.

Finally, since SH_1-blocked HMM rather than SH_1-blocked myosin was used in these experiments, the actin activations of the SH_1-blocked HMM and SH_1-blocked myosin were compared. If the decreased actin activation of the SH_1-blocked HMM compared to normal HMM were due to a reduced rate for some step in the kinetic cycle then the decreased actin activation should be expected to occur with SH_1-blocked myosin as well as with SH_1-blocked HMM. Table III indicates that this is indeed the case. At 22.2 and 45 μM actin concentration both the SH_1-blocked HMM and the SH_1-blocked myosin are activated to the same extent by actin. These results with myosin are consistent with the concept that MalNEt modification affects some step in the kinetic cycle and since all of our measurements indicate that at V_{max} very little SH_1-blocked HMM is bound to actin the step that has been decreased appears to be the rate of conversion of the SH_1-blocked HMM from the refractory state to the nonrefractory state.

Discussion

The sliding filament model of muscle contraction suggests that in vivo there is a cyclic association and dissociation of the myosin heads and actin as the thick and thin filaments slide past each other (Huxley, 1969). There is also some evidence that a similar association-dissociation cycle occurs in vitro during actin activation of the HMM or S-1 ATPase (Eisenberg and Kielley, 1972). The Lymn-Taylor model postulated such a cycle on the basis of data suggesting that the dissociation of the acto-HMM complex

by ATP was faster than the hydrolysis of ATP on the HMM head (Lymn and Taylor, 1971).

Evidence that a cycle occurs in vitro also came from the work of Eisenberg and Kielley. From viscosity, turbidity, analytical ultracentrifuge, and kinetic studies they were able to demonstrate that under conditions where the actin-activated ATPase is close to its maximum value most of the HMM heads remain free of actin (Fraser et al., 1975; Eisenberg et al., 1972a). It is difficult to see how this could occur unless there were a cycle occurring in which the HMM head dissociates from actin after binding a molecule of ATP and then remains in this dissociated or refractory state for most of the cycle until a rate-limiting transition to the nonrefractory state occurs. Binding to actin can then occur followed by product release and dissociation of the acto-HMM complex by ATP.

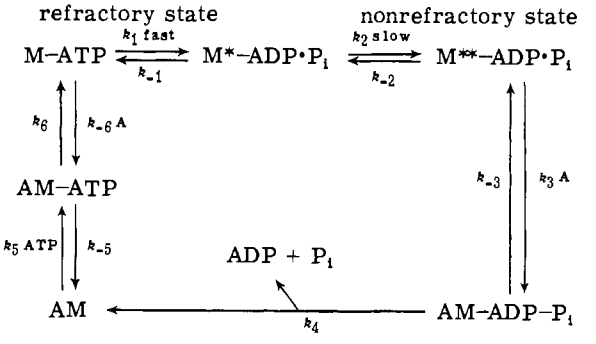
The results presented in this paper suggest that a cycle also occurs with SH_1-blocked HMM. Turbidity and viscosity studies indicate that in the presence of ATP very little interaction between SH_1-blocked HMM and actin occurs under conditions where the binding of actin is not itself rate limiting, i.e. where the actin-activated ATPase nearly equals V_{max}. Binding studies in the analytical ultracentrifuge in the presence of ATP and 25 or 50 m*M* KCl also showed no binding between actin and SH_1-blocked HMM when the ATPase was near V_{max}. In the absence of KCl there was limited binding but even this binding did not appear to correlate with the SH_1-blocked HMM ATPase rate. This binding was very similar in both magnitude and dependence on actin concentration to the limited binding of normal HMM to actin observed in the absence of KCl at 0°. Evidence for this limited binding both for normal and SH_1-blocked HMM was also obtained from viscosity data in the absence of KCl. The nature of this binding remains unclear. It may be due to a nonspecific interaction occurring because of the very low ionic strength or it may in fact be related to the physiologic interaction of actin and HMM. Further work will be required to distinguish between these alternatives.

Data from other laboratories in which myosin was spin labeled at the SH_1 site with a paramagnetic derivative of iodoacetamide also suggested that most of the SH_1-blocked HMM was not bound to actin during ATP hydrolysis. In the absence of ATP the electron spin resonance (ESR) spectrum indicated that the spin-labeled myosin could interact with actin at a ratio of 2 actins to 1 myosin just as the binding data in the analytical ultracentrifuge demonstrated (Stone, 1973; Seidel, 1973). On the other hand the ESR spectrum was not able to detect any interaction between spin-labeled myosin and actin during ATP hydrolysis even though it was clear that the actin was activating the SH_1-blocked HMM ATPase (Stone, 1973; Seidel, 1973).

From all of these data it seems reasonable to conclude that in vitro the SH_1-blocked HMM interacts with actin in a cyclic manner and as with normal HMM the rate-limiting step in the cycle is the transition from the refractory state which cannot bind to actin to the nonrefractory state which is able to bind to actin. If on the contrary the rate-limiting step were product release from the SH_1-blocked HMM–actin complex then almost all of the SH_1-blocked HMM would be expected to be bound to actin when the ATPase equalled V_{max}. But since almost all of the SH_1-blocked HMM was free at V_{max}, the rate-limiting step must be a transition which occurs prior to the rebinding of SH_1-blocked HMM to actin. Furthermore, since the blocking of SH_1 markedly decreases the actin-activated ATPase, presumably this transition is much slower for SH_1-blocked HMM than for normal HMM. Of course this interpretation of our data depends on there being only one species of modified HMM present, i.e. it must be demonstrated that the SH_1-blocked HMM which remains unbound to actin during ATP hydrolysis has the same ATPase activity as the original SH_1-blocked HMM and preliminary studies in this laboratory have suggested that this is indeed the case (Mulhern et al., 1975).

The work of Seidel and Gergely (1973) has demonstrated that myosin blocked with a spin label at SH_1 shows an initial burst of P_i and presumably this is also true for HMM blocked with MalNEt. If in addition, as is true for normal myosin, the initial burst is considerably faster than the steady-state rate in the presence of actin then the rate-limiting transition from the refractory to the nonrefractory state must occur after the initial rapid hydrolysis of ATP. This in turn suggests that the nucleotide bound to myosin in the refractory state has already been hydrolyzed to ADP and P_i. In fact, it is conceivable that it is the hydrolysis of the nucleotide in the initial burst which is itself involved in the formation of the refractory state, a possibility previously suggested for normal HMM by Eisenberg and Kielley (1972). On this basis the following kinetic scheme (Scheme I) could explain our data with SH_1-blocked HMM, where M = SH_1-blocked HMM, (M*-ADP·P_i, M**-ADP·P_i) = SH_1-blocked HMM product complex, and A = actin. In this model the rate-limiting step is k_2 which follows the initial rapid hydrolysis of ATP. SH_1-blocked HMM in the nonrefractory state then interacts with actin which causes rapid release of products. In the next step ATP dissociates the actomyosin complex and the SH_1-blocked HMM returns to the refractory state. The nature of the transition from the refractory to the nonrefractory states remains unclear. It may represent a change in the state of the bound nucleotide on myosin. In the above model the nucleotide is represented as having been hydrolyzed in both the refractory and the nonrefractory states but in fact this is not certain, particularly since Tonomura (1974) has found that only one of the HMM heads shows the initial burst. On the other hand the transition from the refractory to the nonrefractory state may simply represent a conformational change in the myosin head. Tonomura and his colleagues have suggested a conformational change may occur in myosin during ATP hydrolysis (1972). However, their model is quite complex since they postulated two pathways for myosin ATP decomposition with a rate-limiting transformation from $E^0 \rightarrow E$ where E^0 and E represent two different conformations of myosin (1972). At this time we have no evidence for either two different pathways of myosin ATP de-

Scheme I



composition or for the occurrence of a rate-limiting conformational change on myosin without a bound nucleotide.

In conclusion it can be stated that blocking the SH_1 site of myosin does not affect the ability of HMM to bind to actin in the absence of ATP nor does it affect the cyclic interaction of HMM with actin and ATP. On the other hand, the blocking of the SH_1 site of myosin does decrease the actin activation of the HMM ATPase and evidence was presented in this paper that this may be explained by a decreased rate of conversion from the refractory to the nonrefractory state in SH_1-blocked HMM.

References

Daniel, J. L., and Hartshorne, D. J. (1974), *Biochim. Biophys. Acta 347,* 151.

Eisenberg, E., Dobkin, L., and Kielley, W. W. (1972a), *Proc. Natl. Acad. Sci. U.S.A. 69,* 667.

Eisenberg, E., Dobkin, L., and Kielley, W. W. (1972b), *Biochemistry 11,* 4657.

Eisenberg, E., and Kielley, W. W. (1972), *Cold Spring Harbor Symp. Quant. Biol. 37,* 145.

Eisenberg, E., and Moos, C. (1967), *J. Biol. Chem. 242,* 2945.

Eisenberg, E., and Moos, C. (1968), *Biochemistry 7,* 1486.

Fraser, A. B., Eisenberg, E., Kielley, W. W., and Carlson, F. C. (1975), *Biochemistry* (in press).

Gellert, M. F., and Englander, S. W. (1963), *Biochemistry 2,* 39.

Huxley, H. E. (1969), *Science 164,* 1356.

Huxley, H. E., and Brown, W. (1967), *J. Mol. Biol. 30,* 383.

Inoue, A., and Tonomura, Y. (1974), *J. Biochem. 76,* 755.

Kielley, W. W., and Harrington, W. F. (1960), *Biochim. Biophys. Acta 41,* 401–421.

Lymn, R. W., and Taylor, E. W. (1970), *Biochemistry 9,* 2975.

Lymn, R. W., and Taylor, E. W. (1971), *Biochemistry 10,* 4617.

Miller, A., and Tregear, R. T. (1970), *Nature (London) 226,* 1060.

Mulhern, S. A., Eisenberg, E., and Kielley, W. W. (1975), *Fed. Proc., Fed. Am. Soc. Exp. Biol.* (in press).

Seidel, J. C. (1973), *Arch. Biochem. Biophys. 157,* 588.

Seidel, J. C., and Gergely, J. (1973), *Arch. Biochem. Biophys. 158,* 853.

Sekine, T., and Kielley, W. W. (1964), *Biochim. Biophys. Acta 81,* 336.

Silverman, R., Eisenberg, E., and Kielley, W. W. (1972), *Nature (London) 240,* 207.

Spudich, J. A., and Watt, S. (1971), *J. Biol. Chem. 246,* 4866.

Stone, D. B. (1973), *Biochemistry 12,* 3672.

Tonomura, Y., Hyashi, Y., and Inoue, A. (1972), *Cold Spring Harbor Symp. Quant. Biol. 37,* 169.

Yamaguchi, M., and Sekine, T. (1966), *J. Biochem. (Tokyo) 59,* 24.

Young, M. D., Himmelfarb, S., and Harrington, W. F. (1964), *J. Biol. Chem. 239,* 2822.

The Dissociation Constant of the Actin–Heavy Meromyosin Subfragment-1 Complex†

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ABSTRACT: We measured the binding of [^{14}C]iodoacetamide labeled heavy meromyosin subfragment-1 (S-1) to F-actin by sedimenting the actin–S-1 complex and assaying the radioactivity remaining in the supernatant. The apparent dissociation constants (K_d) at 25°, pH 7.0, were 0.01 to 0.04 μM at 0.027 and 0.08 ionic strengths and 0.07 to 0.14 μM at 0.14 ionic strength. K_d was not altered when the troponin–tropomyosin complex was bound on the actin, nor was it affected by free calcium concentration in the range 10^{-4} to 10^{-9} M. Measurements of the displacement of labeled S-1 from actin by native S-1 showed labeling had not altered K_d. In control experiments we found that at the low actin concentrations used (0.01–0.5 μM) not all of the actin sedimented and, furthermore, the data suggested that some of the S-1 in the supernatant was bound to supernatant actin. Our estimation of K_d, based on the assumption that all the supernatant S-1 was free, therefore resulted in an apparent K_d greater than the true K_d. We minimized the effect of the supernatant actin artefact by using only the data for high ratios of S-1 to actin, where no less than 75% of the actin sedimented; we estimate that the true K_d values could not be less than half the apparent K_d values.

Actin and myosin, the major proteins involved in muscle contraction, bind very tightly to each other in the absence of ATP. It has long been believed that the cycling of the myosin bridges in muscle involves the formation and dissociation of these so-called rigor complexes (Huxley, 1969). They most likely represent an intermediate state in actin-activated ATP hydrolysis by myosin. The dissociation constant for the rigor complex is one of the determinants for the overall reaction rate of ATP hydrolysis by actomyosin whose value adds to the kinetic analysis which has made considerable progress in recent years (Kanazawa and Tonomura, 1965; Lymn and Taylor, 1971, Trentham et al., 1972; Bagshaw and Trentham, 1974; Marston, 1973).

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