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Independent and Cooperative Reactions of Myosin Heads with F-Actin in the Presence of Adenosine Triphosphate*

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ABSTRACT: A myosin solution partially inactivated with 6-mercapto-9β-ribofuranosylpurine 5′-triphosphate (SH-ATP) (an analog of ATP) is a mixture of molecules having zero, one, and two of their "heads" enzymatically inactivated [concentrations: $(1 - p)^2C_t$, $2p(1 - p)C_t$, and p^2C_t, respectively, where p = fraction of myosin reacted, C_t = total concentration of myosin]. To discover whether the cooperative reaction of the two heads of myosin is required for contraction, the partially reacted system was compared to a control system consisting of native myosin and myosin totally inactivated with SH-ATP (concentrations: C_n and C_i, respectively; $C_n + C_i = C_t$) with respect to *extent* and *rate* of superprecipitation (E_{spptn} and $\bar{V}_{spptn}$) and actin-enhanced ATPase rate ($\bar{V}_{ATPase}$) in the presence of molar excess actin. E_{spptn}'s reached by reacted systems (p = 0.2, 0.3, and 0.5; C_t = 0.1 mg/ml) and control systems ($C_n + C_i$ = 0.1 mg/ml) are approximately identical (control experiments show $E_{spptn} \propto C_t$). A double-log plot of $\bar{V}_{spptn}$ of the reacted systems *vs.* $(1 - p)^2C_t$ is essentially superimposable on that of $\bar{V}_{spptn}$ of the control systems *vs.* C_n, while a plot of $\bar{V}_{ATPase}$ of the reacted systems *vs.* $(1 - p)C_t$ is superimposable on that of $\bar{V}_{ATPase}$ of the control systems *vs.* C_n. The performance ratio of $\bar{V}_{spptn}$ of a reacted system (p = 0.4, C_t = 0.1 mg/ml) to a control system (C_n = 0.04 mg/ml, C_i = 0.06 mg/ml) observed experimentally (0.61 at 4.6×10^{-5} M ATP) is approximately identical with that calculated (0.73) assuming that the only effective molecules in superprecipitation are those with two native heads and considering that the ratio is equal to the ratio of effective molecules in these two systems raised by power 3 (since control experiments show $\bar{V}_{spptn} \propto C_n^3$ at 4.6×10^{-5} M ATP). However, the observed performance ratio of $\bar{V}_{ATPase}$ (1.4 $\sim$ 1.6) is best explained by considering that all unreacted heads participate in ATP hydrolysis [$(1 - p)^2C_t + 0.5 \times 2p(1 - p)C_t/C_n$ in control system = 1.5]. These results suggest that in catalyzing ATP hydrolysis there is no cooperative reaction between "heads" of the same molecule, but, in order to cause structural change with actin, a myosin molecule must have both of its two heads undamaged.

There is growing evidence that myosin is a duplex molecule with two globular "heads," attached to a rodlike tail, and that each head carries one ATPase site and one actin binding site (Lowey *et al.*, 1967, 1969; Slayter and Lowey, 1967; Schliselfeld and Bárány, 1968; Nauss *et al.*, 1969; Murphy and Morales, 1970). However, the "purpose" of these two heads in muscle contraction has not been examined. In the present work, in order to learn whether the concerted action of these two heads is required in muscle contraction, we attempted to prepare molecules, one head of which is enzymatically inactive.

An ATP analog, 6-mercapto-9-β-ribofuranosylpurine 5′-triphosphate (SH-ATP),[1] is a specific affinity label for the ATPase sites of myosin; 2 moles of this analog attach to 1 mole of myosin, and myosin ATPase activity is thereby reduced to zero. Furthermore, the two ATPase sites of myosin alone appear to be equivalent and noninteracting (Murphy and Morales, 1970). Therefore, a myosin solution partially reacted with SH-ATP will be a mixture of three species of molecules: (1) molecules having two native heads, (2) molecules having one native and one reacted head, and (3) mole-

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[1] Abbreviations used are: SH-ATP, 6-mercapto-9β-ribofuranosylpurine 5′-triphosphate; PK–LDH enzymes, mixed pyruvate kinase and lactic dehydrogenase enzymes; PEP, phosphoenolpyruvate; NEM, *N*-ethylmaleimide; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetate.

cules having two reacted heads. The concentration of these three species can be estimated from simple probabilistic considerations.

In this paper, we have compared a system of myosin partially reacted with SH-ATP to a control system, with respect to reactions of superprecipitation and ATP hydrolysis in the presence of excess actin, *i.e.*, as constituents of synthetic actomyosin. The only effective molecules in superprecipitation were found to be those with two native heads, whereas in ATP hydrolysis, half-labeled molecules also participated.

Materials and Methods

Myosin was obtained by the standard method of our laboratory (Tonomura *et al.*, 1966). Actin was extracted from acetone-dried muscle powder at 0°, and purified by the method of Cohen (1966). Mixed pyruvate kinase and lactic dehydrogenase enzymes (pK–LDH enzymes), phosphoenolpyruvate (PEP), the crystalline sodium salt of adenosine triphosphate (ATP), *N*-ethylmaleimide (NEM), and Tris buffer were obtained from Sigma Chemical Co. Tetramethylammonium chloride was purchased from Southwestern Analytical Chemicals. SH-ATP was synthesized from the barium salt of the monophosphate as described previously (Murphy *et al.*, 1970). Other chemicals used in this work were all reagent grade.

Labeling of myosin with SH-ATP was performed by essentially the same method described by Murphy and Morales (1970). In most experiments, 10 ml of 2% myosin was dialyzed for 24 hr (five changes) against 0.5 M tetramethylammonium chloride–0.05 M Tris (pH 8.2) at 0°. (Replacement of potassium by tetramethylammonium ions reduces the ATPase activity of myosin to nearly zero.) In some experiments, this dialysis was carried out against 0.6 M NaCl–0.1 M $NaHCO_3$ (pH 8.2). To 7 ml of 1.4% myosin in either of these solvents was added a 400-, 200-, or 160-fold excess of SH-ATP per 4.8 $\times$ 10^5 g of myosin dissolved in 3 ml of the same solvent (mol wt 684 for SH-ATP and 4.8 $\times$ 10^5 for myosin).

To follow the decay of the ATPase rate, at an appropriate time interval after addition of SH-ATP, a 0.1-ml aliquot of sample was withdrawn and poured into 14.9 ml of solution of 0.6 M KCl–8 mM $CaCl_2$–1.15 mM ATP–0.05 M Tris (pH 8.2), and the ATP hydrolysis rate was measured at 25°. When the ATPase activity decreased to an appropriate value, 2 or 3 ml of treated myosin was layered on a column of DEAE-Sephadex A-50 (2 $\times$ 25 cm) equilibrated with 0.3 M KCl–0.05 M histidine (pH 7.0) and eluted with the same solution at a flow rate of 60 ml/hr in a 0° cold room. The myosin solution so eluted was further dialyzed overnight in the cold room against 0.6 M KCl–0.05 M histidine (pH 6.5). A half-reacted or a totally reacted solution of myosin was obtained by removing excess SH-ATP as described above, when the ATPase activity of the myosin was approximately half the original or was reduced to nearly zero, respectively.

The stoichiometry of the labeling was determined from the difference in the optical density at 322 nm of SH-ATP myosin after addition of excess β-mercaptoethanol and control myosin at pH 6.5, assuming that for unbound SH-ATP, ϵ_{322} = 23,100 (Hampton and Maguire, 1961).

Actomyosin solution was prepared by mixing actin and myosin in 0.3 M KCl. After allowing it to stand for 30 min on ice, the KCl concentration was reduced to 0.05 M by adding an appropriate buffer solution. Partially reacted actomyosin was synthesized from actin and partially reacted myosin.

Superprecipitation was measured by following the change in optical density at 660 nm as a function of time. The extent of superprecipitation was expressed as the maximum increment in turbidity and the rate of superprecipitation is shown as the extent divided by $\tau_{0.5}$, which is the time required for the turbidity to reach half-maximum. To maintain the ATP concentration constant, a pyruvate kinase–PEP system was coupled with the actomyosin ATPase.

ATPase activity was measured by the Fiske–Subbarow method. Protein concentration was determined by a modified Folin–Ciocalteau procedure.

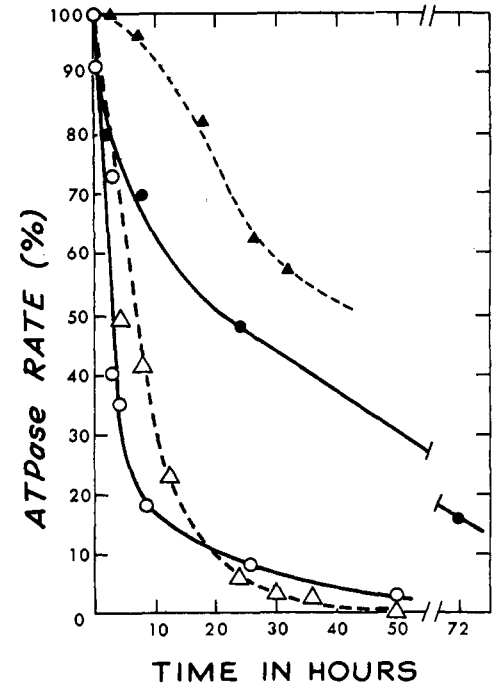

FIGURE 1: Time course of the ATPase rate upon incubation with excess SH-ATP. Conditions for incubation are 0.5 M $(CH_3)_4NCl$–0.05 M Tris (pH 8.2), 0°. 400-fold molar excess SH-ATP (△), 200-fold molar excess SH-ATP (▲) per 4.8 $\times$ 10^5 g of myosin was added to myosin immediately after dissolving in buffer solution. 200-fold molar excess SH-ATP (○) and 160-fold molar excess SH-ATP (●) per 4.8 $\times$ 10^5 g of myosin were allowed to stand overnight at 0° before adding to myosin. Conditions for ATPase assay are 0.6 M KCl·8-mM $CaCl_2$–1.15 mM ATP·0.05 M Tris (pH 8.2), 25°.

Results

Labeling of Myosin with SH-ATP. The time course of decay of the myosin ATPase activity with incubation with SH-ATP is affected by preincubation of the SH-ATP in buffer solution (Figure 1). If SH-ATP is mixed with myosin immediately after dissolving it in buffer solution, the time course of the decay of ATPase activity shows a lag phase of about 30 min or 3 hr when incubation is with a 400- or 200-fold molar excess of SH-ATP per 4.8 $\times$ 10^5 g of myosin, respectively. When SH-ATP is allowed to stand in buffer solution overnight before adding it to myosin, no initial lag is observed, and the decay rate is much faster than that obtained without preincubation of the SH-ATP, even when a lower excess molarity of SH-ATP is employed.

The amounts of SH-ATP bound to myosin measured by β-mercaptoethanol reduction were 1.16 and 2.01 moles of SH-ATP per 4.8 $\times$ 10^5 g of myosin for a half-reacted and a totally reacted myosin, respectively; thus the extent of inhibition of the ATPase activity is a linear function of the SH-ATP bound to myosin.

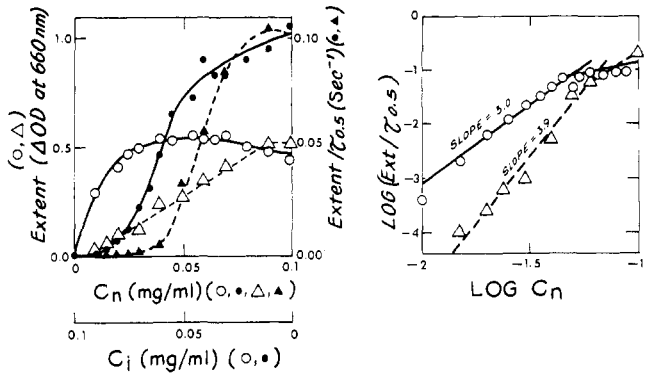

FIGURE 2: Myosin concentration studies. (A) Extent and rate of superprecipitation *vs.* myosin concentration. Conditions are 0.1 mg/ml of actin–0.05 M KCl–1 mM $MgCl_2$–0.046 mM ATP–0.1 mM $CaCl_2$–0.09 mM EGTA–0.5 mM PEP–0.1 mg/ml of PK–LDH enzyme–0.02 M Tris-maleate (pH 7.0), 25°. Concentration of native myosin is varied from 0.01 to 0.10 mg per ml (△, ▲). Concentration of native myosin is varied from 0.01 to 0.10 mg per ml, and in the same solution concentration of totally reacted myosin is varied from 0.10 to 0.01 mg per ml, in such a way that the total concentration of myosin is constant for all points (○, ●). C_n is the concentration of native myosin and C_i is the concentration of totally reacted myosin. (B) Double-log plot of superprecipitation rate *vs.* native myosin concentration. System has two species of myosin molecule—doubly native and doubly dead (○), or native molecules only (△). The points were obtained from the data shown in part A.

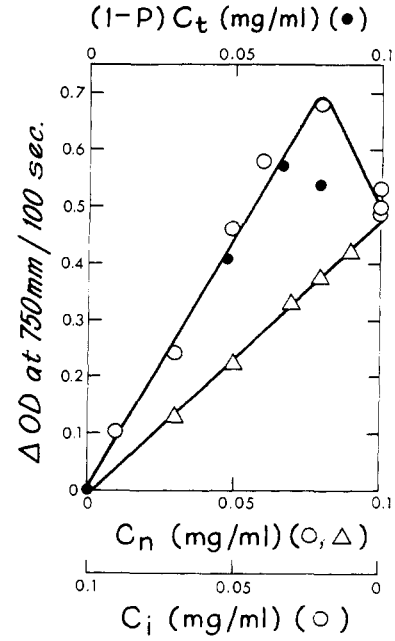

FIGURE 4: Dependence on the myosin concentration of the initial rate of actomyosin ATPase. Conditions for ATPase assay and explanation of ○ and △ are the same as described in Figure 3. The ATPase rate of partially reacted actomyosin (●) is plotted *vs.* $(1 - p)C_t$, where p is the fraction of myosin reacted with SH-ATP, and C_t is the total concentration of partially reacted myosin. The points were obtained from data shown in Figure 5B.

The ATPase activity of the totally reacted myosin can be restored to levels which are 90–100% of the original by adding excess β-mercaptoethanol.

Dependence of the Rate and Extent of Superprecipitation and of the Actomyosin ATPase Rate on the Myosin Concentration When Actin is in Excess. Prior to the experiments using partially reacted myosin, we inquired how the rate and extent of superprecipitation or the actin-activated ATPase rate depend on myosin concentration. These experiments were carried out using two different systems. (I) Known concentrations of native myosin from 0.01 to 0.1 mg per ml were mixed with 0.1 mg of actin/ml. (II) In the presence of 0.1 mg of actin/ml, known concentrations of native myosin from 0.01 to 0.10 mg per ml were mixed with known concentrations of totally reacted myosin in such a way that the *total* myosin concentration remained constant (0.10 mg/ml).

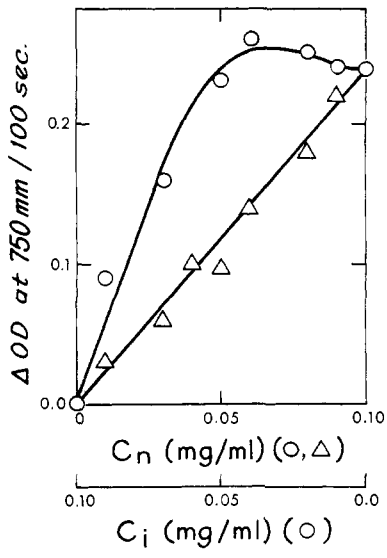

FIGURE 3: Dependence on the myosin concentration of the steady state ATPase rate of actomyosin. System contains doubly native and doubly dead myosin molecules (○) or native molecules only (△). Conditions are 0.1 mg/ml of actin–0.1 mg/ml of PK–LDH enzyme–1 mM PEP–0.2 mM ATP–1 mM $MgCl_2$–0.1 mM $CaCl_2$–0.09 mM EGTA–0.05 M KCl–0.02 M Tris-maleate (pH 7.0), 25°.

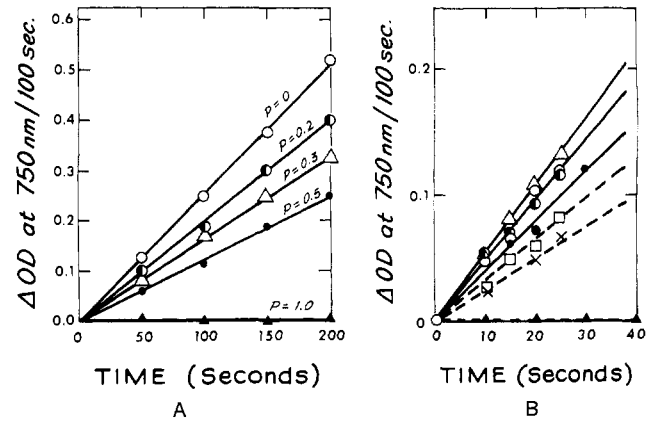

FIGURE 5: Time course of ATP hydrolysis. (A) Under standard conditions by myosin partially reacted with SH-ATP. Myosin was incubated with 160-fold molar excess SH-ATP per 4.8×10^5 g of myosin for 0 hr (○), 2 hr (◐), 3 hr (△), 24 hr (●), and 140 hr (▲). p is the fraction of myosin reacted. Conditions for ATPase assay; 0.067 mg/ml of myosin–0.6 M KCl–8 mM $CaCl_2$–1.15 mM ATP–0.05 M Tris (pH 8.2), 25°. (B) By actomyosin synthesized from partially reacted myosin and actin. Treated system contains 0.1 mg/ml of actin and 0.1 mg/ml of myosin reacted with SH-ATP for 2 hr (◐), 3 hr (△), 24 hr (●), and 140 hr (▲). Control system contains 0.1 mg/ml of actin and 0.1 mg/ml (○), 0.07 mg/ml (□), and 0.05 mg/ml (×) of native myosin. Experimental conditions are the same as described in Figure 4.

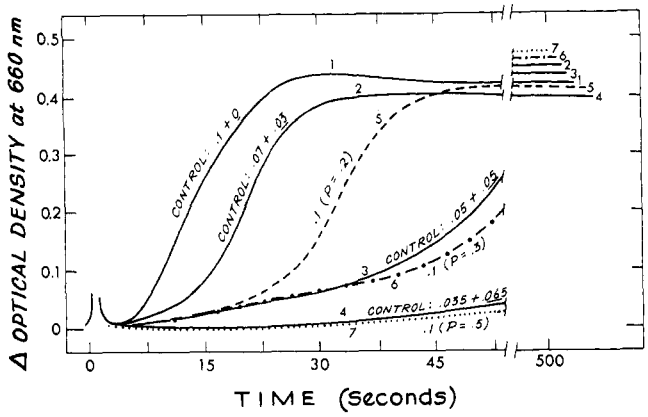

FIGURE 6: Time course of superprecipitation. Control actomyosin systems contain 0.1 mg/ml of actin and (1) 0.1, (2) 0.07, (3) 0.05, (4) 0.035 mg per ml of native myosin and (1) 0, (2) 0.03, (3) 0.05, (4) 0.065 mg per ml of totally reacted myosin. Reacted systems contain 0.1 mg/ml of actin and 0.1 mg/ml, of (5) "20%-reacted", (6) "30%-reacted," and (7) "50%-reacted" myosin. Conditions are 0.1 mg/ml of PK–LDH enzyme–0.5 mM PEP–0.2 mM ATP–1 mM $MgCl_2$–0.1 mM $CaCl_2$–0.09 mM EGTA–0.05 M KCl–0.02 M Tris-maleate (pH 7.0), at 25°.

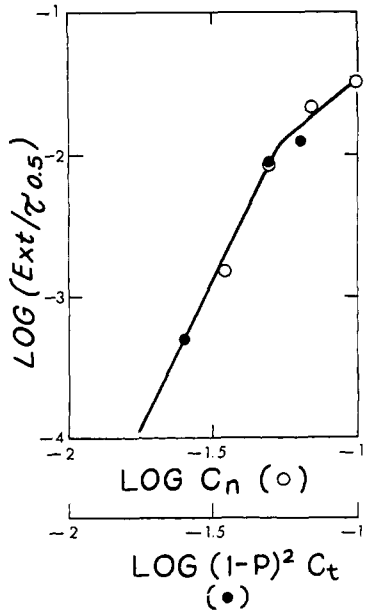

FIGURE 7: Double-log plot of superprecipitation rate *vs.* concentration of doubly native myosin or $(1 - p)^2C_t$ in a treated system, and a superimposed double-log plot for a control system in which the concentration of doubly native molecules is $C_n = (1 - p)^2C_t$ and the concentration of doubly dead molecules is $C_t - (1 - p)^2C_t$.

As shown in Figure 2A, in an experiment using system I, the extent of superprecipitation is strictly proportional to the myosin concentration, from 0.01 to 0.09 mg per ml. On the other hand, using system II, the extent is nearly constant in the range of *native* myosin concentration from 0.025 to 0.10 mg per ml, and it decreases below 0.02 mg of native myosin/ml. The superprecipitation rate is higher in the presence than in the absence of totally reacted molecules. However, only totally reacted myosin and actin do not superprecipitate.

Figure 2B shows a plot of log (superprecipitation rate) *vs.* log (native myosin concentration), in the presence and absence of totally reacted myosin. These plots show that the superprecipitation rate obeys the following empirical relations below a native myosin concentration of 0.05 mg/ml.

Rate of superprecipitation $\propto$ (native myosin)$^{\omega}$, where ω is 3.9 or 3.0, in the case of system I or II, respectively.

Figure 3 shows the dependence on the myosin concentration of the steady state ATPase rate. In the absence of totally reacted myosin, the ATPase rate is strictly proportional to the native myosin concentration. On the other hand, using system II, the ATPase rate increases linearly with native myosin concentration in the range from 0.01 to 0.06 mg per ml, but, in the range from 0.06 to 0.10 mg per ml, the rate is approximately independent of myosin concentration. The initial rate of ATPase in the presence of some totally reacted myosin shows a nonlinear dependence on the native myosin concentration, exhibiting a maximum at a concentration of 0.08 mg of native myosin/ml; however, the rate is strictly proportional to native myosin concentration in the absence of totally reacted molecules (Figure 4).

ATPase and Superprecipitation of Actomyosin Reconstituted from SH-ATP Myosin and Excess Actin. Figure 5A shows the time course of ATP hydrolysis by myosin reacted with SH-ATP for different times. p is the fraction of myosin reacted with SH-ATP. Figure 5B shows the time course of ATP hydrolysis of actomyosin reconstituted from the myosin preparation shown in Figure 5A. The rates of ATP hydrolysis of these SH-ATP actomyosins are much higher than those of control actomyosin systems having the same concentrations of native heads. In Figure 4, the ATPase rates of these treated systems are plotted *vs.* $(1 - p)C_t$, where C_t is the total concentration of treated myosin. In the same figure, the ATPase rates of a control system containing some totally reacted myosin are plotted against the concentration of native myosin. As is evident from this figure, the two plots approximately superimpose.

The time course of superprecipitation using the same solution of SH-ATP myosin as in the experiments of Figure 4A,B is shown in Figure 6 together with those of control systems. The extents of superprecipitation reached by these systems are almost identical within experimental error. In Figure 7, the logarithm of the superprecipitation rate of control and reacted systems is plotted *vs.* log C_n and log $(1 - p)^2C_t$, respectively,

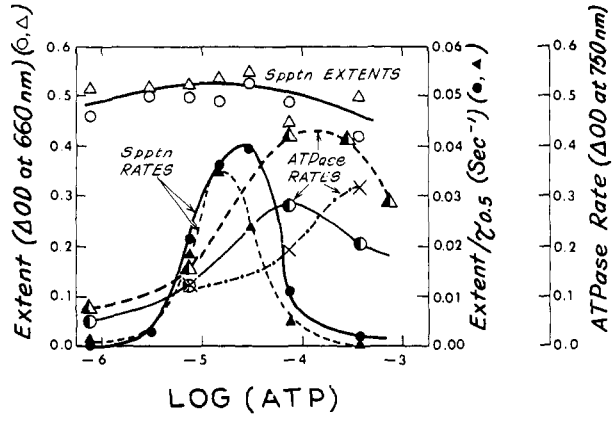

FIGURE 8: Plot of steady-state ATPase rate, extent, and rate of superprecipitation *vs.* log of ATP concentration. (○, △) Extent of superprecipitation. (●, ▲) Rate of superprecipitation. (◐, ◭, ×) Rate of ATPase. Control system contains 0.1 mg/ml of actin, 0.04 mg/ml of doubly native myosin, and 0.06 mg/ml of doubly dead myosin, (○, ●, ◐), or 0.1 mg/ml of actin and 0.06 mg/ml of doubly native myosin (×). Treated system contains 0.1 mg/ml of actin and 0.1 mg/ml of "40%-reacted" myosin (△, ▲, ◭).

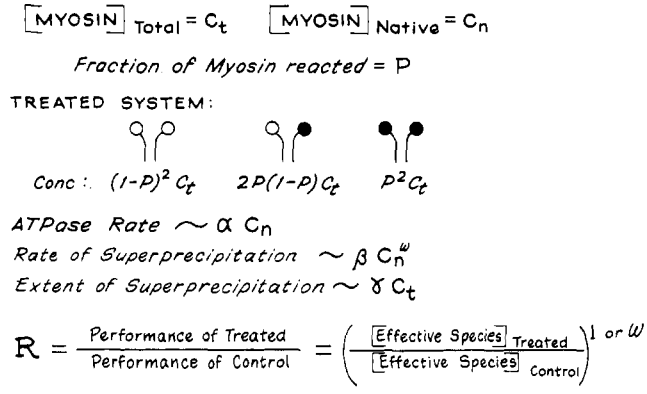

FIGURE 9: Strategy of our experiments with mixtures of various species of the myosin molecule (see text).

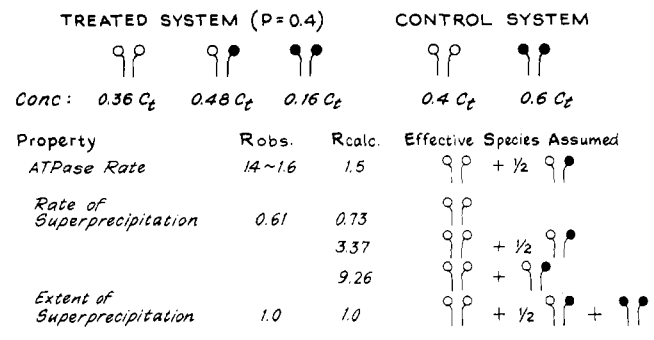

FIGURE 10: The performance ratio of treated and control system (see text). Data shown in Figure 8 were used.

where C_n is the concentration of native myosin. As is shown in this figure, the two plots essentially superimpose.

In Figure 8, the rate and extent of superprecipitation and the steady state ATPase rate of "40% reacted" actomyosin are shown as a function of "feeder"-maintained ATP concentration. As a control, reactions of actomyosin reconstituted from 40% native myosin and 60% totally reacted myosin are shown. The extents of superprecipitation reached by these two systems are almost identical and approximately constant in the range of ATP concentration used. On the other hand, the rate of superprecipitation varies as a function of ATP concentration, and shows a maximum at 2×10^{-5} M ATP for the treated system and at 4.6×10^{-5} M ATP for the control system. The maximum ATPase rate of the reacted system is 1.4 ~ 1.6 times that of control, and this ratio is approximately the same as the ratio of the fractions of unreacted "heads" of myosin in these two systems. However, the ATPase rate of the reacted system at 7×10^{-5} M ATP is 2.2 times that of the control system reconstituted from actin and 60% native myosin only, even though the former has the same concentration of native heads as the latter.

Discussion

When SH-ATP is mixed with myosin immediately after dissolving it in buffer, a lag in the decay of the myosin ATPase activity is observed, and this lag phase is eliminated by preincubating the SH-ATP solution on ice overnight. It therefore seems likely that, as in the case of many other SH reagents (Hartshorne and Morales, 1965), SH-ATP binds to myosin by an exchange reaction between the SH group of myosin and a disulfide formed from SH-ATP.

A solution of myosin partially reacted with SH-ATP is the mixture of molecules having zero, one, and two of their heads enzymatically inactivated. In the light of present evidence, it is reasonable to assume that in the absence of actin the two heads of myosin react *independently* with a reagent such as SH-ATP. Murphy and Morales (1970) studied the binding of SH-ATP when it was acting as a substrate (not as an affinity label), using the slight absorbance change upon binding in conjunction with stopped flow apparatus. Their binding data, analyzed in "Scatchard fashion" plots as a straight line, are suggesting only one affinity constant, *i.e.*, suggesting that the two heads bind SH-ATP independently. Such independence has also been reported in the case of other analogs, *e.g.*, ADP (Lowey and Luck, 1969). Furthermore, although our data are somewhat scattered and sometimes complicated by a lag phase (see above), the decay of ATPase upon incubation time with SH-ATP suggests only one rate constant (roughly logarithmic decay), and there is a linear relationship between the number of unreacted sites and the ATPase. Consequently, the concentrations of the three possible species of molecules in an SH-ATP myosin solution can be estimated from simple probabilistic considerations (Figure 9). Then knowing how the ATPase rate, and the rate and extent of superprecipitation of actomyosin depend on the concentration of myosin, we can discover which species of molecules are effective in ATPase or in superprecipitation.

As is shown in Figure 4, the plot of the ATPase rate of the treated system *vs.* $(1 - p)C_t$, which is the concentration of native heads, is practically superimposable on the plot of the control system. This means that not only molecules with two native heads but those with one native and one inactive head in some way participate in catalyzing ATP hydrolysis. Another implication is that there is no evidence of cooperation between "heads" on the same "stem" as regards catalysis.

In contrast with this result, the only effective molecules as regards superprecipitation rate are those with both heads native. The plot of log of the superprecipitation rate *vs.* log of $(1 - p)^2 C_t$, which is the log of the concentration of totally native molecules in the treated system, is essentially superimposable on the plot of the control system containing totally reacted molecules. From this result, we can say that, as regards superprecipitation rate, half-reacted molecules behave like totally reacted molecules. Although both types of molecules accelerate the rate of superprecipitation, half-reacted molecules alone will not cause this reaction, since a totally reacted myosin–actin system does not superprecipitate.

In Figure 10, the ratios of the performance of the reacted system to that of the control system have been calculated by considering that in one reaction only one species—or a particular combination of species—is effective, and that the performance ratio is equal to the ratio of the concentrations of effective molecules raised to an appropriate power—one in the case of the ATPase rate and three in the case of the superprecipitation rate. Then, we have compared this calculated ratio to the ratio measured experimentally. In the experimental system shown in Figure 8, the concentration of molecules having zero, one, and two of their heads enzymatically inactivated is $0.36C_t$, $0.48C_t$, and $0.16C_t$, respectively, since $p = 0.4$, and the control system is the mixture of $0.4C_t$ of native molecules and $0.6C_t$ of totally reacted molecules. As is evident from this figure, concerning superprecipitation extent, doubly labeled molecules and half-labeled molecules contribute about as well as doubly native molecules. The molecular events of superprecipitation are not yet quite clear, but it is not surprising

that enzymatically dead molecules participate in producing extent, because reacted molecules can probably form thick filament-like aggregates together with native molecules and interact with actin, as long as their rodlike portions and actin binding sites remain intact. On the other hand, our data suggest that in order to bring about *structural* change with actin, a myosin molecule must have *both* of its heads undamaged. Specifically in the case of superprecipitation rate, the performance ratio at 4.6×10^{-5} M ATP concentration is observed to be 0.61. This ratio is best explained by assuming that, as regards superprecipitation rate, the only effective molecules are those having two native heads, since

$$\left[\frac{(\text{native molecules})_{\text{reacted system}}}{(\text{native molecules})_{\text{control system}}}\right]^3 = \left[\frac{0.36}{0.40}\right]^3 = 0.73$$

As regards the ATPase rate, the performance ratio is observed to be $1.4 \sim 1.6$. If we assume that all unreacted heads participate in ATP hydrolysis, the ratio is expected to be 1.5. From these results, it is concluded that two "heads" of myosin are functioning independently in catalyzing ATP hydrolysis even in the presence of actin.

It is suggestive for future work that in contributing to superprecipitation rate—*i.e.*, to the rate of a structure-changing reaction— *both* heads of the *same* molecule must be native in order for that molecule to be effective. Such a requirement would be natural if the reaction consisted in some form of cooperative movement of the two heads on actin. Additional evidence for inter-head cooperation on actin has been reported by Cooke and Smith (1970).

Two important problems remain unresolved. (1) Enzymatically inactive molecules labeled with SH-ATP accelerate the superprecipitation rate, when they are mixed with normal actomyosin, despite the fact that actomyosin synthesized from totally reacted myosin and actin does not superprecipitate. (2) The ATPase rate of normal actomyosin is enhanced by adding myosin molecules totally inactivated with SH-ATP.

Currently we are attempting to develop methods of independently counting actin–myosin contacts; in the absence of such a technique our view of these unresolved problems has to be speculative. For example, we may imagine that in the chain of events during the structural change in actin–myosin–ATP system (Takahashi and Yasui, 1967) there are two fundamental reactions: a rate-limiting formation of nuclei or clusters (Tada and Tonomura, 1967), followed by some action in which only effective types of myosin molecules participate, *e.g.*, doubly native for motion or any native head for ATPase. We could then say that at a constant concentration of effective molecules the rate of superprecipitation will nevertheless be observed to depend on, say, doubly dead molecules, since the rate of nuclear formation and the number of nuclei formed will depend on the concentration of "stem" or rod portion of myosin molecules. In addition, at a constant concentration of "stem"—that is, the total concentration of myosin molecules—the rate of superprecipitation will depend exactly on the concentration of doubly native molecules.

It might be thought that stimulation of actomyosin ATPase by totally inactive myosin might be due to dilution of SH-ATP block by transfer (disulfide exchange). However, since actomyosin reconstituted from actin and a mixture of native myosin and inactive myosin treated with NEM which does not transfer is also found to exhibit high ATPase activity, this possibility seems highly unlikely.

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