Bunting, 1974), so that K_{SS}/K_{SS}' may be considerably less than 1 and yet still lead to $K_S K_{SS}/K_S' K_{SS}'$ being significant relative to 1. Thus, the presence of even small amounts of S_2EH_2 and S_2EH relative to the concentration of EHS_2 may lead to a dependence on $pK_{S_2EH_2}^{app}$ for k_3^{app}. It is clear that $(1 + K_S K_{SS}/K_S' K_{SS}')$ cannot be very much greater than 1 for **1c,** otherwise the apparent k_3/k_{OH} ratio would be much less than the range of values observed for **1d–g.**

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Pre-Steady-State Kinetic Evidence for a Cyclic Interaction of Myosin Subfragment One with Actin during the Hydrolysis of Adenosine 5′-Triphosphate

Stephen P. Chock,* P. Boon Chock, and Evan Eisenberg

ABSTRACT: A single cycle of adenosine 5′-triphosphate (ATP) hydrolysis by a complex of actin and myosin subfragment one (acto-S-1) was studied in a stopped-flow apparatus at low temperature and low ionic strength, using light scattering to monitor the interaction of S-1 with actin and fluorescence to detect the formation of fluorescent intermediates. Our results show that the addition of a stoichiometric concentration of ATP to the acto-S-1 causes a cycle consisting of first, a rapid dissociation of the S-1 from actin by ATP; second, a slower fluorescence change in the S-1 that may be related to the initial phosphate burst; and third, a much slower rate limiting recombination of the S-1 with actin. This latter step equals the acto-S-1 steady-state adenosine 5′-triphosphatase (ATPase) rate at both low and high actin concentrations, and like the steady-state ATPase levels off at a V_{max} of 0.9 s^{-1} at high actin concentration. Therefore, the release of adenosine 5′-diphosphate and inorganic phosphate is not the rate-limiting step in the acto-S-1 ATPase. Rather, a slow first-order step corresponding to the previously postulated transition from the refractory to the nonrefractory state precedes the rebinding of the S-1 to the actin during each cycle of ATP hydrolysis.

It is now generally accepted that contraction of skeletal muscle is caused by the relative sliding of the thick myosin filaments past the thin actin filaments, and considerable evidence suggests that this sliding process is driven by the cyclic interaction of cross bridges extending from the myosin filament with actin and ATP (Huxley, 1969; Huxley, 1974). Clearly in vivo, by its very nature, this interaction must involve not only the association but also the dissociation of the cross bridge from actin during each cycle of ATP hydrolysis. The first evidence that dissociation and reassociation of the actin and myosin occurred each time ATP was hydrolyzed in vitro came from the work of Lymn and Taylor (1971; Taylor, 1972). Their pre-steady-state experiments showed that when ATP was added to a complex of actin and heavy meromyosin (HMM), dissociation of the actin–HMM complex by ATP occurred before the P_i burst, or before the initial burst of ATP hydrolysis on the HMM head. On this basis, they proposed the cycle shown in Scheme I for ATP hydrolysis (where A and M represent actin and myosin, and T, D, and P_i represent ATP, ADP, and phosphate, respectively). The major rate-limiting step in this cycle was a relatively slow release of products occurring

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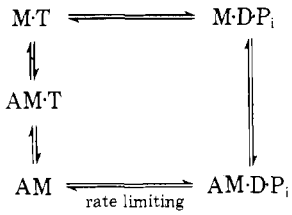
‡ Abbreviations used are: S-1, myosin subfragment-one; acto-S-1, a complex of actin with S-1; HMM, heavy meromyosin; ATP, adenosine 5′-triphosphate; P_i, inorganic phosphate; OD, optical density.

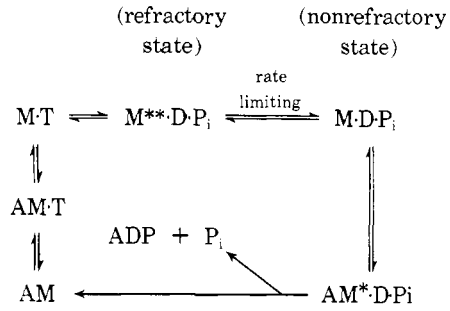
Scheme I



after the HMM rebound to actin. Thus, this model as originally presented suggested that at high actin concentration, a large fraction of the HMM or S-1 would remain complexed with actin even in the presence of ATP (but cf. Lymn, 1974).

Evidence that a cycle of dissociation and reassociation of actin and myosin occurs in vitro each time ATP is hydrolyzed also came from the work of Eisenberg and Kielley (1972). Numerous studies on the actin activation of the HMM or S-1 ATPase showed that over a wide range of temperature and ionic strength, there was a simple hyperbolic relationship between ATPase and F-actin concentration (Eisenberg and Moos, 1970). However, viscosity, turbidity, quasi-electric light scattering, analytical ultracentrifuge, and kinetic studies all demonstrated that under conditions where the actin-activated ATPase was close to its maximal value, a large fraction of the HMM or S-1 remained dissociated from the actin. These results were not consistent with the Lymn–Taylor model (Eisenberg et al., 1972; Fraser et al., 1975). To explain these Eisenberg and Kielley (1972) proposed the cycle in Scheme II. In this cycle, as in the Lymn–Taylor model, binding of ATP

Scheme II



to the hydrolytic site caused dissociation of the myosin head. However, in contrast to the Lymn–Taylor model, the myosin head remained in this dissociated or refractory state for most of the cycle, until a rate limiting transition to the nonrefractory state occurred. Only then did rebinding to actin occur which in turn caused rapid release of products. Thus, in contrast to the slow product release suggested by the Lymn–Taylor model, the major rate-limiting step in the Eisenberg and Kielley model was the transition from the refractory to the nonrefractory state that occurred before the myosin head could rebind to actin.

In regard to the cycles proposed by Lymn and Taylor, and Eisenberg and Kielley, several basic questions arise. First, is this general type of cycle correct, i.e., do the dissociation and reassociation of the actin and S-1, in fact, occur each time an ATP molecule is hydrolyzed? Many other kinds of ATPase cycle are possible—two ATP molecules may be hydrolyzed during each dissociation and reassociation cycle, or two kinds of S-1 head may occur, each undergoing a different cycle (Tonomura, 1966). In addition, it is always possible that the first cycle observed when ATP is added is different from the succeeding cycles due to slow ATP-induced changes in the conformation of the myosin.

If indeed dissociation and reassociation of the actin and S-1 occurs each time an ATP is hydrolyzed, the next question that arises is the nature of the rate-limiting step in the cycle. At low actin concentration presumably binding to actin is rate limiting but at high actin concentration the rate-limiting step could be the step involving product release that occurs after actin binding as proposed by Lymn and Taylor (Scheme I), or it could be a step involving transition from the refractory to the nonrefractory state that occurs before actin binding as proposed by Eisenberg and Kielley (Scheme II). Finally, if the latter is the case, the question arises as to the nature of the refractory and nonrefractory states and, in particular, how they relate to the intermediates observed when myosin hydrolyzes ATP in the absence of actin.

Several laboratories have previously investigated a single cycle of interaction of actomyosin with ATP. At high ionic strength where no actin activation occurs, Finlayson et al. (1969) found that following dissociation of the actomyosin by stoichiometric ATP, the actin and myosin reassociated at a rate about equal to the steady-state ATPase rate of the myosin alone. On the other hand, working at lower ionic strength where actin activation occurs, both Inoue et al. (1973) and Hozumi and Tawada (1974) found that the rate of reassociation of the HMM–substrate complex with actin was considerably slower than the steady-state actin-activated ATPase rate. Working with the S-1–substrate complex, Hozumi and Tawada did find that the maximum rate of reassociation of the actin and S-1 was similar to (one-half) the maximum actin-activated ATPase rate, but, unfortunately, these experiments were performed at 0.2 M KCl where little actin activation occurs at experimentally obtainable actin concentrations and thus extrapolation to infinite actin concentration is difficult.

In the present study we investigated a single cycle of interaction of acto-S-1 with ATP, using light scattering as a measure of binding between the actin and S-1. We also studied fluorescence changes to help determine the nature of the intermediates that occurred during the cycle. We worked at very low ionic strength so that the actin-activated ATPase could be brought close to its maximal value, and at 5 °C so that the measured rates would be relatively slow and thus could be more accurately measured. We used S-1 rather than HMM because presumably the binding of a single-headed species will be simpler than the binding of a two-headed one.

Our results show that under these conditions, addition of stoichiometric ATP to acto-S-1 causes a cycle consisting of first, a rapid dissociation of the acto-S-1 complex by ATP; second, a somewhat slower fluorescence change in the dissociated S-1–substrate complex; and third, a much slower rate limiting recombination of the S-1–substrate complex with actin. Our results also show that over a tenfold range of actin concentration, the rate of recombination of the S-1–substrate complex with actin equals the steady-state ATPase rate and most important, like the ATPase rate, levels off at high actin concentration. These results show that in contrast to the Lymn–Taylor model, the S-1–substrate complex undergoes at least two different conformational changes before it can rebind to actin. The first causes a fluorescence change and may be related to the initial P_i burst. The second is about tenfold slower than the fluorescence change, and equals the maximum actin-activated ATPase rate and thus may represent the rate limiting transition from the refractory to the nonrefractory state previously proposed by Eisenberg and Kielley.

Materials and Methods

Protein Preparation. Mysoin was prepared from rabbit skeletal muscle using the method of Kielley and Harrington

(1960). S-1 was prepared from myosin using soluble papain as described by Lowey et al. (1969), and dialyzed against 2 mM imidazole, pH = 7, buffer. F-actin was prepared using a modified method of Spudich and Watt (1971). To make sure that tropomyosin and troponin were removed, the F-actin was twice suspended in 0.8 M KCl (Eisenberg and Kielley, 1974) instead of just once with 0.6 M KCl as originally described by Spudich and Watt. The final F-actin solution contained 2 mM $MgCl_2$, 10 mM imidazole, and 0.5 mM ATP. The ATP was removed using Dowex-1 anion-exchange resin as described by Mulhern et al. (1975). The molecular weight of S-1 was taken as 120 000, and that of actin as 43 000. The imidazole used is of fluorometric grade from Sigma.

Absorbance as Measurements of Turbidity. The absorbance measurement was done at 350 nm using a Cary-14 spectrophotometer thermostated at 5 °C. The reaction was started by mixing 3.8 ml of actin solution containing 13 μM actin, 8.7 mM imidazole, 2 mM $MgCl_2$, 0.47 mM K_2HPO_4, and 2 mM ATP with 1.2 ml of S-1 solution containing various S-1 concentrations in 2 mM imidazole buffer. The resulting protein mixture contained 7.1 mM imidazole, 1.5 mM $MgCl_2$, 0.4 mM K_2HPO_4, and 1.5 mM ATP. The turbidity of the acto-S-1 in the presence of ATP is very close to the sum of the turbidities of the actin and S-1 measured alone (Fraser et al., 1975); therefore, the difference in absorbance between the initial state of the reaction when ATP was present and the final absorbance after all the ATP had been hydrolyzed was taken as a measure of the turbidity of the acto-S-1 complex.

Stopped-Flow Apparatus. The stopped-flow apparatus used here essentially consists of a mercury-xenon arc light source powered with a Kepco JQE power supply, a Bausch and Lomb grating monochromator equipped with a manual lens shutter and adjustable slits, a thermostated two-syringe Aminco-Morrow stopped-flow pneumatic drive system equipped with a modified mixer flow cell (Chock and Eisenberg, 1974) the temperature of which was directly read via a Teflon probe attached to the outer wall of the observation flow cell and registered on a Yellow Spring Instrument tele-thermometer, an EMI 9558 QBS-20 photomultiplier tube powered by a Keithley 245 high-voltage supply, a fast interacting signal amplifying system (Rhee and Chock, 1976), and a Tektronix WP-1200 digital processing system where waveform averagings and computations were carried out.

Stopped-Flow Experiments. All stopped-flow experiments were performed under the same experimental conditions: no KCl, 7.8 mM imidazole, 1.5 mM $MgCl_2$, pH 7, 5 °C. The acto-S-1 solution was made up by careful dropwise addition of a concentrated S-1 solution to a stirring actin solution. Air bubbles were removed from the solution meniscus after centrifugation in a desk top centrifuge at medium speed. The ATP solution was also made up to contain the same buffer. All concentrations given represent the reaction chamber concentration, and all stopped-flow traces represent a computer average of four oscilloscope traces obtained from four consecutive runs of the same reaction.

In the fluorescence experiments, an excitation wavelength of 300 nm was used. The 90 degree fluorescence emitted light was passed through a Corion interference filter that has a peak transmission (15%) at 340 nm and half-peak band of 10 nm before reaching the photodetector. When turbidity was measured, a wavelength of 340 nm was used. The 90 degree scattered light was also passed through the same Corion interference filter. This way we could measure both the turbidity and fluorescence changes in the same reaction by simply changing the monochromator setting.

Treatment of Data. Each averaged waveform was digitized, stored, and analyzed in the DEC-PDP-11/05 computer. For a first-order reaction the waveform was analyzed according to the equation

$$\ln \frac{(V_\infty - V_t)}{(V_\infty - V_0)} = -kt$$

where V_∞, V_t, and V_0 are relative millivolt readings at the end of the reaction, at time t, and at the beginning of the reaction, respectively. The rate constant k is then calculated from the slope of the plot of log $[(V_\infty - V_t)/(V_\infty - V_0)]$ vs. t. For a second-order reaction, such as the binding of S-1 to actin that is essentially irreversible, the simple second-order reaction equation

$$\frac{1}{a-b} \ln \frac{b(a-x)}{a(b-x)} = kt$$

was used to derive the following equation

$$\frac{1}{a-b} \ln \frac{\left(b - \frac{bx}{a}\right)}{(b-x)} = kt$$

where a and b are initial concentrations, x is the concentration of product at time t, and k is the second-order rate constant. From the above equation an equation suitable for our stopped-flow experiments was derived:

$$\frac{1}{a-b} \ln \frac{(V_\infty - V_0) - \frac{b}{a}(V_t - V_0)}{V_\infty - V_t} = kt$$

where V_∞, V_t, and V_0 are as defined above. The value of k was then directly calculated from the slope of the line obtained by plotting the left-hand side of the equation vs. t.

Results

To observe a single cycle of interaction of ATP with acto-S-1, measurements must be made on a millisecond time scale and, at the present time, the only parameters related to the binding between actin and S-1 that have been measured on this time scale are light scattering and absorbance. Finlayson et al. (1969) used abosrbance as a measure of binding in stopped-flow experiments and have given a theoretical basis for their measurements. On the other hand, as pointed out by Fraser et al. (1975), in a complex system like acto-HMM or acto-S-1 interpretation of light scattering measurements is complicated by the interaction between actin filaments that may take place.

If light scattering and absorbance were a linear measure of binding between actin and S-1, then in the absence of ATP they should be proportional to the acto-S-1 concentration. Figure 1 shows the OD at 350 nm of a solution of 10 μM F-actin as a function of increasing S-1 concentration. We performed this study at very low ionic strength at 5 °C, the conditions that we planned to use in our subsequent experiments on a single cycle of ATP hydrolysis. As can be seen under these conditions, turbidity does not appear to be a strictly linear measure of binding between the actin and S-1. Rather the curve is slightly concave upward, although it does level off at close to a stoichiometric ratio of S-1 to actin, suggesting that the binding is relatively strong under this condition. One possible explanation for the concave shape of the curve is that at relatively high ratios of S-1 to actin, increasing actin-actin interaction leads to gel formation and extra turbidity. This explanation might also account for the results of preliminary stopped-flow ex-

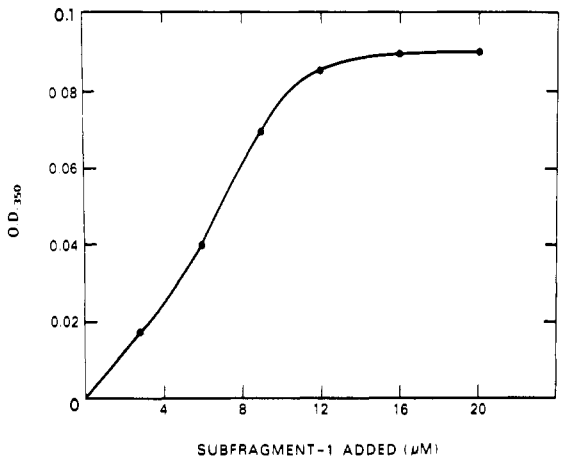


FIGURE 1: Turbidity as a measure of acto-S-1 binding. The change in absorbance at 350 nm was measured when 10 μM of actin was titrated with various concentrations of S-1 as described under the Materials and Methods section.

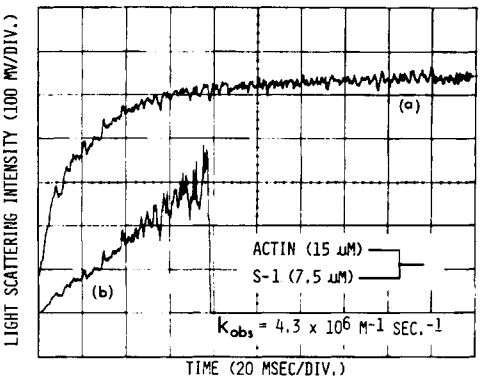


FIGURE 2: Kinetics for the formation of acto-S-1 complex. Curve (a) represents the time course of the turbidity changes at 340 nm following the mixing of actin with S-1 as measured in the stop-flow apparatus using light-scattering technique. Curve (b) represents the computer semilog plot of (a) analyzed as a simple second-order reaction as described under Materials and Methods. One syringe contained actin (15 μM, reaction chamber concentration), and the other syringe contained S-1 (7.5 μM, reaction chamber concentration). Both syringes also contained 7.8 mM imidazole, 1.5 mM $MgCl_2$, pH 7, 5 °C. The k_{obsd} in this reaction equals 4.3×10^6 M^{-1} s^{-1}.

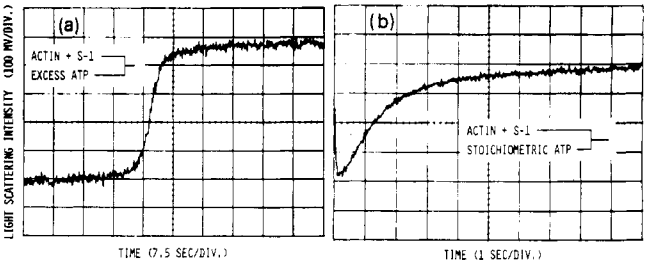


FIGURE 3: Turbidity changes following the addition of ATP to acto-S-1 complex. (a) The time course of turbidity changes following the addition of excess ATP (300 μM) to acto-S-1 complex. There is a rapid drop in turbidity at the beginning of the reaction followed by a regain in turbidity after all the ATP has been consumed. (b) The time course of turbidity changes following the addition of a stoichiometric amount of ATP (10 μM) to acto-S-1 complex (10 μM). The initial drop in turbidity is immediately followed by the regain in turbidity. Other than the difference in the concentration of ATP added, both (a) and (b) were performed under the same experimental conditions. One syringe contained acto-S-1 (40 μM actin + 10 μM S-1), the other syringe contained ATP. Other conditions are the same as in Figure 2.

periments carried out at 25 °C at stoichiometric ratios of S-1 to actin. Under this condition, just mixing the acto-S-1 in one syringe with buffer in the other caused a small transient decrease in light scattering quite possibly because the shear during mixing transiently broke up the acto-S-1 gel. At ratios of actin to S-1 of 2:1 or greater at 5 °C this phenomenon did not occur and thus these higher ratios were used in all of our subsequent experiments.

One test of whether light scattering is a reasonably good measure of binding between actin and S-1 is to use light scattering to study the rate of binding in the absence of ATP. The binding should be a simple second-order reaction and thus if the scattered light intensity is directly proportional to the acto-S-1 concentration, the second-order semilog plot should be linear. As can be seen in Figure 2, this indeed appears to be the case. As in all the stopped-flow data presented in this paper, the curve shown in Figure 2 is a computer average of four runs (see Methods). The apparent second-order rate constant for the binding of S-1 to actin is about 5×10^6 M^{-1} s^{-1}, which is not too far from the diffusion-limited rate constant that might be expected for this reaction. Thus, based on the linearity of our second-order plot, we can conclude that under the conditions where we will investigate the single cycle of actin-S-1 interaction with ATP, light scattering appears to be a relatively good measure of binding between actin and S-1.

Before investigating a single cycle of interaction of acto-S-1 with stoichiometric ATP, we investigated what occurred when excess ATP was added. Fraser et al. (1975) have already demonstrated in steady-state experiments that, in the presence of up to 3 mM ATP and under the conditions of maximal actin activation of the S-1 ATPase, the turbidity of the acto-S-1 is very close to the value expected in the absence of any interaction between the actin and S-1, but that it rises to a much higher value after all of the ATP is hydrolyzed. Figure 3a shows that a similar result occurs in the stopped-flow apparatus. On addition of 100 μM ATP to acto-S-1 a rapid drop in light scattering occurs. The light scattering then remains at a constant low value until the ATP is all hydrolyzed at which time a rapid rerise in light-scattering occurs. Since the rate of ATP hydrolysis is essentially linear during the course of the reaction, the length of time from the initial drop in turbidity until its rerise to half of its maximum turbidity is proportional to the ATP concentration. Therefore, the total added ATP divided by this length of time can be used as a measure of the steady-state ATPase rate and this method gives a value quite similar to the ATPase rate determined by the pH-stat method. Thus, we have the advantage of being able to perform both our pre-steady state and steady state kinetic measurement in the same apparatus under identical experimental conditions. This is particularly important for our experiments because it is difficult to thermostat the stopped-flow cell at exactly 5 °C and the acto-S-1 ATPase is very sensitive to temperature changes.

Figure 3b shows that, when stoichiometric ATP is added to the acto-S-1, a rapid dissociation of the acto-S-1 occurs followed by a much slower reassociation. Comparison of the light scattering changes in Figure 3b with those in Figure 3a shows that the dissociation is about 80% complete which means that during the observed cycle almost every S-1 molecule hydrolyzes one ATP molecule and very few S-1 hydrolyze more than one ATP. This in turn means that we are effectively observing a single cycle of ATP hydrolysis. In preliminary experiments with ATP less than stoichiometric, the magnitude of the dissociation is less but the rates of both the dissociation and

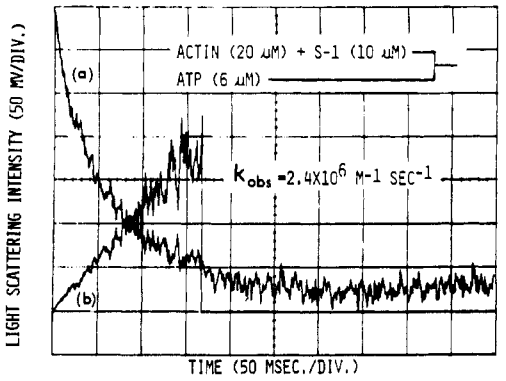


FIGURE 4: Dissociation of acto-S-1 complex by ATP. Curve (a) represents the time course of the turbidity changes following the addition of ATP to acto-S-1 complex. Curve (b) represents the computer semilog plot of (a) analyzed as a bimolecular reaction. One syringe contained acto-S-1 complex (20 μM actin + 10 μM S-1), and the other syringe contained ATP (6 μM). Other conditions are the same as described in Figure 2. The k_{obsd} in this reaction equals 2.4×10^6 M^{-1} s^{-1}.

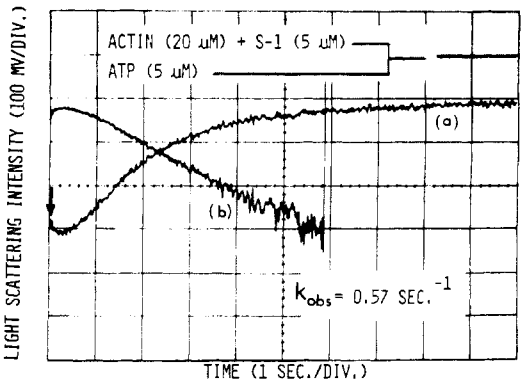


FIGURE 5: Kinetics of actin-S-1 reassociation following the hydrolysis of a stoichiometric concentration of ATP. Curve (a) represents the time course of the turbidity changes following the addition of a stoichiometric concentration of ATP to acto-S-1 complex. The arrow marks the fast initial drop in turbidity that was too fast to be recorded at this time scale. The kinetics of this initial drop is shown in Figure 4. Curve (b) represents the computer semilog plot of (a) from which a pseudo-first-order rate constant, k_{obsd} (= 0.57 s^{-1}), was obtained. See Materials and Methods for details. One syringe contained acto-S-1 complex (20 μM actin + 5 μM S-1), and the other syringe contained ATP (5 μM). Other experimental conditions are the same as in Figure 2.

reassociation process corresponds well to the case when a stoichiometric ATP is added. With ATP greater than stoichiometric, of course, a delay in the rerise in turbidity develops. It therefore appears that with stoichiometric ATP, we are observing a single cycle of ATP hydrolysis and the crucial question is how the rates observed for the steps in the cycle relate to the steady-state ATPase rate.

We began our study of the single ATPase cycle by investigating the rate of dissociation of the acto-S-1 by ATP. Figure 4 shows that the dissociation process gives a linear second-order semilog plot with an apparent second-order rate constant equal to 2.4×10^6 M^{-1} s^{-1}. Therefore, like the binding of actin to S-1, the dissociation of the acto-S-1 by ATP is a quite rapid process. Of course, as shown by Lymn and Taylor, the dissociation of the acto-S-1 by ATP must be at least a two-step process involving the second-order binding of ATP followed by a first-order dissociation of the ternary acto-S-1-ATP complex. However, the rate constant for the latter step, which determines the maximum rate of dissociation, is so large that it cannot be obtained experimentally. Therefore, under our conditions, as the ATP concentration is increased, the second order rate constant remains constant, i.e., the rate of dissociation linearly increases with increasing ATP concentration. This result agrees with the findings of Lymn and Taylor at 20 °C and 50 mM KCl (Lymn and Taylor, 1971).

Having shown that under our conditions, the dissociation of the acto-S-1 by ATP followed simple second-order kinetics and was quite rapid, we turned to an investigation of the reassociation of the actin and S-1. This process too might be thought to be second-order, but in these experiments we always used a 3 to 4-fold molar excess of actin over S-1 and thus could analyze it as a pseudo-first-order reaction. Figure 5 shows a typical plot of the rerise in turbidity analyzed in this way. As can be seen, after a slight lag during the early part of the reaction, the rerise in turbidity follows simple first-order kinetics giving a linear semilog plot from which a pseudo-first-order rate constant can be obtained. We could therefore study the dependence of the first-order rate constant on actin concentration and compare it with the steady-state ATPase rate.

The first point we noted was that, as shown in Figure 5 for 20 μM actin, and at all actin concentrations studied, the reassociation process appeared to go to completion in a single monophasic reaction. It is not surprising that the reaction went to completion because the cycle starts with completely complexed acto-S-1 and must end the same way. However, the rebinding could occur in two steps, a rapid partial rebinding followed by a slower reassociation to give a completely complexed acto-S-1. In fact, we did occassionally observe a very small amount of slow upward creep in the turbidity following the end of the first-order process, but this was probably due to a very small amount of slow gelling occurring following the reassociation of the actin and S-1. Therefore, the overall process itself appears to go essentially to completion in a single monophasic process.

We next compared the rate of reassociation of the actin and S-1 with the steady state ATPase rate at various actin concentrations. Over a tenfold range of actin concentration, the pseudo-first-order rate constant for the reassociation process measured under the single turnover condition and the steady-state ATPase rate measured in the stopped-flow apparatus as described above agree within 20% of each other (Figure 6). Several additional experiments with different protein preparations gave this same result. Since the single turnover experiment measures the rate at which S-1 can rebind to actin and the steady-state experiment measures the overall rate around the cycle, from the above results, it would appear that the rate limiting step around the cycle is the rebinding of S-1 to actin.

It is not surprising that the rebinding of S-1 to actin should be rate limiting at low actin concentration, since under this condition, doubling either the S-1 or actin concentration doubles the ATPase rate, a typical behavior for a second-order binding reaction. On the other hand, at high actin concentration doubling the actin no longer doubles the ATPase rate. Rather, both the steady-state ATPase rate and the rate of reassociation of the actin and S-1 seem to level off at a maximum value. This hyperbolic relationship is clearly demonstrated in Figure 6 in the form of a double-reciprocal plot. The double-reciprocal plot is linear, showing that at high actin concentration the rate of rebinding of S-1 to actin reaches a maximum value of about 0.9 s^{-1}. This maximum rate is much lower than the rate of actin-S-1 binding that would occur in the absence of ATP even at very low actin concentration (see Figure 2). Therefore, the fact that the rate for the reassociation of the actin and S-1 during the ATPase cycle reaches a maxi-

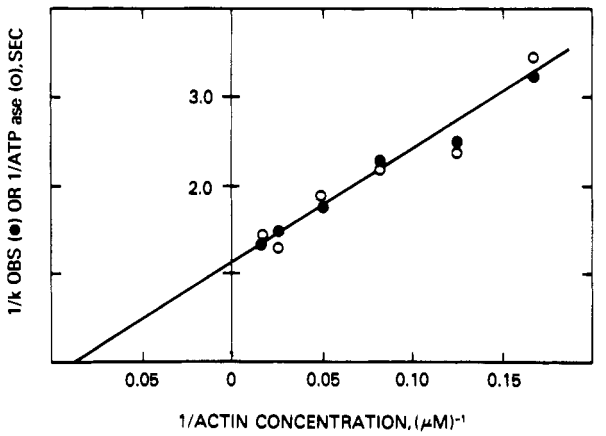


FIGURE 6: Comparison of the steady-state acto-S-1 ATPase rate with the pseudo-first order rate observed for actin-S-1 reassociation during a single turnover of ATP. The open circles (○) represent the steady state ATPase rate measured in the stop-flow apparatus using excess ATP, e.g., Figure 3a. The closed circles (●) represent the pseudo-first-order rate observed for the reassociation of S-1 to actin after hydrolysis of a stoichiometric concentration of ATP, e.g., Figure 5. The ordinate intercept yields a V_{max} of 0.9 s^{-1} for both the steady state ATPase and the reassociation of S-1 to actin after a single turnover of ATP. Experimental conditions are as described under Materials and Methods.

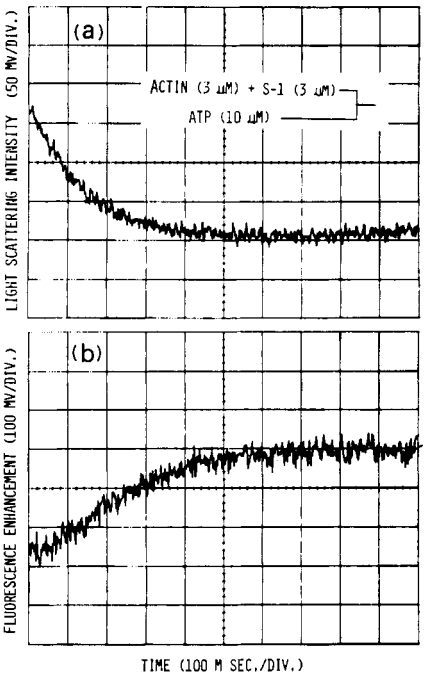


FIGURE 7: Amplitudes of turbidity and fluorescence changes in the dissociation of acto-S-1 with low ATP. (a) Shows the relative amplitude and time course of the turbidity changes. (b) Shows the relative amplitude and time course of the fluorescence changes of the same experiment. One syringe contained acto-S-1 (3 μM actin + 3 μM S-1) and the other syringe contained ATP (10 μM). Other conditions are described in Figure 2.

mum rate is not due simply to some limitation in the second-order rate at which the actin and S-1 can bind to each other; rather, at high actin concentration, some first-order rate limiting process must occur prior to the second-order rebinding of the S-1 to actin. This is in agreement with the earlier suggestion of Eisenberg and Kielley (1972) that at high actin concentration, the first-order transition from the refractory to the nonrefractory state of S-1 becomes the rate-limiting step in the actin-activated ATPase cycle. Thus, the reassociation of S-1 with actin appears to be a two-step process involving first, the transition from the refractory to the nonrefractory state and second, the actual rebinding of the S-1 to the actin.

The next question that arises is the nature of the refractory and nonrefractory states. Considering the ATPase activity of myosin alone, the data of Bagshaw et al. (1974) and Koretz and Taylor (1975) as well as data from our lab (Chock and Eisenberg, 1974, 1975) suggest that the binding of ATP to S-1 occurs in two steps: formation of a collision intermediate followed by a conformational change in the protein. Such a two-step substrate binding process is common, occurring with many enzymes. Following the binding of ATP, however, a step peculiar to myosin occurs in which the ATP is rapidly hydrolyzed in an initial phosphate burst (Kanazawa and Tonomura, 1965) to an intermediate that Bagshaw et al. (1974) have suggested has a somewhat higher fluorescence than the myosin-ATP complex. Then, following this initial phosphate burst, a rate limiting transition to the simple myosin-product complex occurs followed by dissociation of the products (Trentham et al., 1972). Using the notation of Bagshaw et al. (1974) the model for the myosin ATPase is therefore

$$M + T \rightleftharpoons M \cdot T \rightleftharpoons M^* \cdot T \rightleftharpoons M^{**} \cdot D \cdot P_i \rightleftharpoons M + D + P_i$$

where the number of asterisks represents the relative magnitude of the fluorescence of the particular intermediate. The question then is which, if any, of these intermediates is formed when acto-S-1 is dissociated by ATP. One way of approaching this question is to study the fluorescence change that occurs when ATP dissociates acto-S-1.

Figure 7a shows the light-scattering change that occurs when 10 μM ATP is mixed with 3 μM acto-S-1 and Figure 7b shows the accompanying fluorescence change. As can be seen, a significant fluorescence change occurs but its magnitude is difficult to quantitate because three separate phenomena may contribute to it. The first is the fluorescence change caused by the binding of ATP to the S-1, the second is the fluorescence change that might be caused by dissociation of the actin from the S-1, and the third is the light-scattering change that because of the spread in the bandwidth of the incoming light may be reflected to some extent in the fluorescence measurement. To determine the fluorescence change that is due to the binding of ATP itself we investigated the fluorescence change that occurs when acto-S-1 is dissociated by a high ATP concentration.

As noted above, the rate of dissociation of the acto-S-1 by ATP linearly increases with increasing ATP concentration so that at high ATP concentration the dissociation is essentially completed during the deadtime of the stopped-flow apparatus. Therefore, fluorescence changes observed when high ATP concentrations are used will, of necessity, occur *after* dissociation of the acto-S-1 and will not be obscured by changes in light scattering. In this way it should be possible to determine whether M·T, M*·T or M**·D·P_i is formed when acto-S-1 dissociates. If M·T were formed the subsequent fluorescence change would be identical to that observed with S-1 alone in the absence of actin, a fluorescence change that we find occurs relatively slowly even at high ATP concentration. On the other hand, if M**·D·P_i were formed then almost no fluorescence change would be observed, since all of the fluorescence change would occur simultaneously with the dissociation of the acto-S-1 and before the measurement begins. If M*·T were formed then a fluorescence change would be observed as M**·D·P_i forms, but it would presumably be smaller than that for S-1 alone.

Figure 8a shows that when 100 μM ATP is mixed with

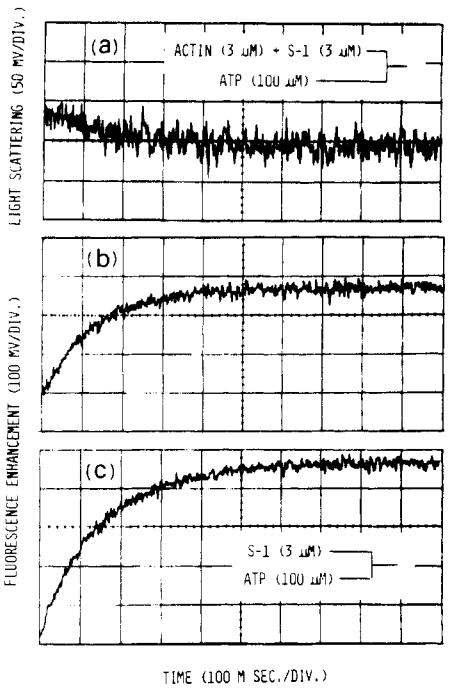


FIGURE 8: Amplitudes of turbidity and fluorescence changes in the dissociation of acto-S-1 by high concentration of ATP. (a) Shows the relative amplitude and the time course of turbidity changes due to the dissociation of acto-S-1 by a high concentration of ATP. (b) Shows the relative amplitude of the fluorescence changes of the same reaction. The k_{obsd} for this reaction was about 7.95 s^{-1}. (c) Shows the relative fluorescence amplitude and time course when the same concentration of ATP is added to S-1 alone. (The k_{obsd} for this reaction was calculated to be 7.3 s^{-1}.) Experimental conditions are the same as in Figure 7 except in (c) where no actin was present.

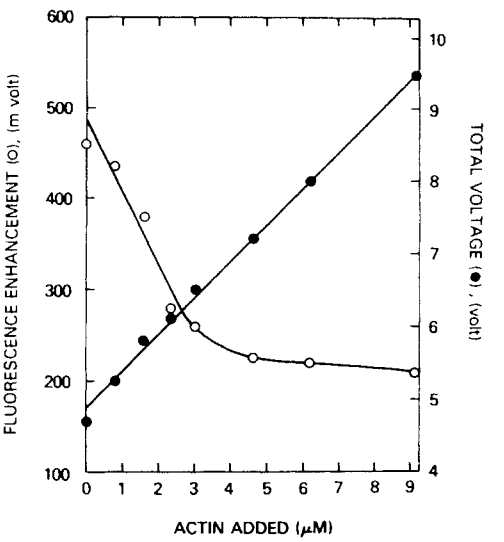


FIGURE 9: Amplitude of the fluorescence change occurring after acto-S-1 dissociation as a function of actin concentration. The open circles (○) represent the relative amplitude of the fluorescence change that occurs following the addition of 100 μM ATP. The closed circles (●) represent the total fluorescence of the reacting system. One syringe contained acto-S-1 (3 μM S-1 + varying concentration of actin), and the other syringe contained ATP (100 μM). Other conditions are as described in Figures 2 and 8.

acto-S-1 the light-scattering change and thus the acto-S-1 dissociation is almost completed before the measurement begins (cf. Figure 7a). On the other hand, as shown in Figure 8b, a significant increase in fluorescence occurs following the acto-S-1 dissociation; and its magnitude is similar to the magnitude of the fluorescence change occurring at low ATP concentration (Figure 7b). Figure 8c shows the fluorescence change that occurs when ATP is mixed with S-1 in the absence of actin. Comparison of Figure 8B and C shows that the presence of actin reduced the magnitude of the fluorescence change about 40% but had only a small effect on its rate. This same result is also observed at higher ATP.

Figure 9 confirms that the effect of actin in reducing the magnitude of the fluorescence change is specific. Here we have plotted as a function of actin concentration the magnitude of both the total fluorescence (total voltage) and the fluorescence enhancement that occurs on addition of ATP. The total fluorescence linearly increases with increasing actin concentration, as would be expected, of course, since the total protein concentration is increasing. On the other hand, the magnitude of the ATP induced fluorescence change decreases as the actin concentration is increased, and even more important, levels off at the equimolar ratio of 1 mol of actin monomer/mol of S-1. Such leveling off at a stoichiometric binding ratio suggests that the effect of actin on the fluorescence change is specific. The slight downward drift in fluorescence at higher actin concentration may be due to nonspecific scattering of the incoming light, i.e., a filter effect as a result of increasing actin concentration. We can therefore conclude that a significant fluorescence change occurs in the S-1 following dissociation of the acto-S-1 by ATP but that the magnitude of this change is significantly less than the change occurring with S-1 and ATP, alone. Based on the ATPase scheme given above, this suggests that acto-S-1 is dissociated by ATP into M*·T and actin, and the subsequent transition from M*·T to M**·D·P_i may be responsible for the fluorescence change we observe following dissociation of the acto-S-1. It is interesting to note that if this interpretation is correct, it means that M*·T forms more rapidly when ATP binds to acto-S-1 than when it binds to S-1 alone.

Discussion

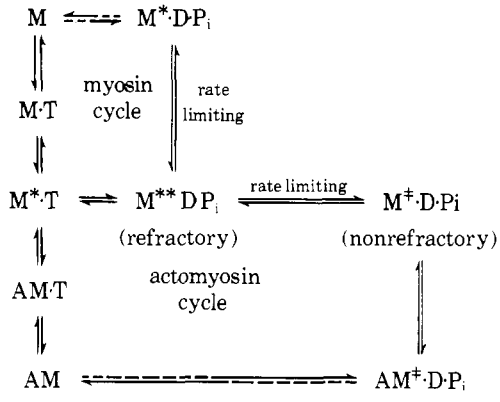
The data presented in this paper show that under conditions where actin strongly activates the S-1 ATPase the rate around a single cycle of ATP hydrolysis in vitro is quite close to the steady-state ATPase rate. This strongly implies that the acto-S-1 dissociates and reassociates each time an ATP molecule is hydrolyzed in vitro by acto-S-1. White and Taylor (personal communication) have recently observed that at 50 mM KCl a similar cycle occurs with acto-HMM. The cross-bridge model of muscle contraction requires that as the thick and thin filaments slide past each other, a cyclic dissociation and reassociation of the myosin cross-bridge and actin occurs. That such a cycle also occurs in vitro when ATP is hydrolyzed lends support to the cross-bridge model of muscle contraction and also suggests that the in vitro behavior of acto-S-1 reflects the behavior of the myosin cross-bridges and actin in vivo.

Of course, it must be emphasized that the cycle described in this paper is based on the assumption that light scattering is a reasonably linear measure of binding between actin and S-1 and its corollary that the binding of nucleotide to the acto-S-1 complex does not markedly *decrease* its light scattering unless the actin and S-1, in fact, dissociate. The concave shape of the curve presented in Figure 1 shows that light scattering is not a strictly linear measure of binding. On the other hand, based on the increase in molecular weight, alone, it seems unlikely that significant binding of the S-1-nucleotide complex to actin could occur without causing an increase in light scattering. Furthermore, ultracentrifuge and kinetic

studies also suggest dissociation is occurring and thus agree with the light-scattering measurement (Eisenberg et al., 1972; Eisenberg and Kielley, 1972). Therefore, we feel reasonably confident that the light-scattering changes that we observe reflect the dissociation and reassociation of the acto-S-1.

Based on the data presented in this paper, the following

Scheme III



model for the acto-S-1 ATPase can be developed. It must be emphasized that this model is not presented as a final answer but simply as a way of illustrating the minimum number of steps necessary to explain our data. As far as possible, we have used intermediates previously proposed by other workers, adding as few new ones as possible. We have also kept the myosin ATPase scheme of Bagshaw et al. (1974) intact, neither adding or removing any intermediate, although more work will be necessary to determine if it is indeed correct. It should be noted that at 5 °C, the rate-limiting step in the actomyosin cycle is about 30 times faster than the rate-limiting step in the myosin cycle.

It is important to distinguish between two separate aspects of this model. The first is the series of steps that is required by our experimental data, for example, the occurrence of a rate limiting conformational change in S-1 following its dissociation from actin. The second is the specific chemical nature of the states involved and their identification with previously proposed myosin–nucleotide intermediates. Here of course the evidence is much weaker.

The first point to be noted in this model is that a fluorescence change occurs in the S-1 simultaneously with its dissociation from actin, that is, using the notation of Bagshaw et al. it is M*·T rather than M·T that forms when ATP dissociates the acto-S-1. This is based on our finding that the fluorescence change that occurs when acto-S-1 is dissociated by high ATP concentration is significantly less than the fluorescence change that occurs when ATP binds to S-1, alone (Figure 9). Presumably, part of the ATP induced fluorescence change on the S-1 occurs at the same time as the rapid dissociation of the acto-S-1 by the high ATP concentration and is thus not detected. At low ATP concentration where this lost fluorescence change might be detected, the magnitude of the fluorescence change is obscured by the dissociation process itself. If actin, like ATP, can induce a fluorescence change in S-1, i.e., AM*, then its dissociation from the S-1 will cause a decrease in fluorescence and this might cancel out the fluorescence change caused by M·T $\rightleftharpoons$ M*·T at low ATP. In addition, light-scattering changes might interfere with the fluorescence measurement. However, from our data at high ATP concentration, we can conclude that ATP induces a conformational change in the S-1 simultaneously with its dissociation from actin. Furthermore, at high ATP concentration this conformational change occurs, i.e., M*·T forms, much more rapidly when ATP binds to acto-S-1 than when ATP binds to S-1 alone. This may be related to the situation in vivo where presumably during the contractile cycle, ATP binds to actomyosin rather than to myosin.

It is not surprising that dissociation of the two strongly bound proteins, actin and S-1, requires an ATP-induced conformational change. The binding of ATP to S-1 to form M*·T is almost irreversible, i.e., shows a very large drop in free energy (Bagshaw and Trentham, 1973; Wolcott and Boyer, 1974; Mannherz et al., 1974), and presumably this free energy is used to dissociate the acto-S-1. On the other hand, the rather weak binding of ATP to S-1 in the collision intermediate, MT, would probably not provide enough free energy to dissociate the acto-S-1. Based on somewhat different data Sleep and Taylor (personal communication) have recently come to a similar conclusion.

Following the formation of M*·T, our data show that at least two steps occur before the S-1 rebinds to the actin. The first of these steps is manifested by a fluorescence change that occurs immediately after the acto-S-1 dissociation, and that has a first-order rate constant about ten times faster than the maximum actin-activated ATPase rate. Since Lymn and Taylor (1971) found that the initial burst of ATP hydrolysis occurred immediately after dissociation of the acto-S-1 and, at least at 20 °C and 50 mM KCl, had a first-order rate constant about ten times faster than the maximum steady-state actin-activated ATPase rate, we have assumed that the fluorescence change we observe is due to the initial P_i burst. This choice is also consistent with the deduction by Bagshaw et al. (1974) based on experiments with ATP analogues, that M**·D·P_i may have a greater fluorescence than M*·T, thus accounting for the fluorescence change that we observe.

Of course it must be emphasized that, although our data show that a conformational change occurs when ATP dissociates acto-S-1, the intermediate formed need not be M*·T. It could be some as yet uncharacterized S-1 nucleotide intermediate. Likewise, the rapid fluorescence change that occurs next need not be caused by the P_i burst. Simultaneous studies of the fluorescence change and the P_i burst will be necessary to determine if they are indeed both caused by the same conformational change.

In any case, following this first conformational change on the dissociated S-1, our data strongly imply that there is a second much slower conformational change on the S-1 before it can rebind to actin. This conformational change represents the transition from the refractory to the nonrefractory state, first proposed by Eisenberg and Kielley (1972) from steady-state data, as the rate-limiting step in the acto-S-1 ATPase cycle. Direct evidence for this step now comes from our pre-steady-state observation that the rate of reassociation of the actin and S-1 levels off at high actin concentration at a maximum rate equal to the maximum actin-activated ATPase rate. If the rebinding consisted of only one second-order step, then its rate would linearly increase with increasing actin concentration and would not level off. This is because we are using light scattering as a measure of binding and presumably the light scattering will detect the presence of the first acto-S-1 species to form even if it is just a collision intermediate. Furthermore, because the rate of binding of S-1 to actin is so rapid in the absence of ATP, the leveling off of the rate of binding in the presence of ATP could not be due to some physical limitation in the rate at which actin and S-1 can bind. Therefore, the hyperbolic relationship between the rate of actin-S-1 rebinding and the actin concentration strongly implies that

there are two steps involved in the reassociation process, one of which occurs before the S-1 rebinds to the actin.

On this basis, and also taking into account that at all actin concentrations studied, the reassociation process seems to be essentially irreversible, the following mechanism is the simplest that can explain our data

$$M^{**}\cdot D\cdot P_i \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} M^{\ddagger}\cdot D\cdot P_i \underset{k_{-2}}{\overset{Ak_2}{\rightleftarrows}} AM^{\ddagger}\cdot D\cdot P_i$$

where $k_{-1} \gg k_1$ and k_{-2} is negligible. With increasing actin, the rate of rebinding approaches $k_1/[1 + (k_{-1}/k_2A)]$, a hyperbolic expression where the maximum rate of rebinding ($V_{max} = 0.9\ s^{-1}$) is k_1 and the *apparent* dissociation constant ($K_{app} = 1.2 \times 10^{-5}$ M) that is clearly *not* a true equilibrium constant is k_{-1}/k_2.

A more complex mechanism that might also explain our data would be to make the binding of $M^{\ddagger}\cdot D\cdot P_i$ to actin reversible, i.e., k_{-2} quite large, but then make some subsequent step, for example, conversion of $AM^{\ddagger}\cdot D\cdot P_i$ to $AM\cdot D\cdot P_i$ or the dissociation of ADP and the P_i, rapid and irreversible. Further work will be necessary to distinguish between these mechanisms and in particular to determine if k_{-1} is larger than k_1, i.e., if the equilibrium between the refractory and nonrefractory state is shifted toward the refractory state.

In our model, we have tried not to add any new states to the myosin ATPase scheme of Bagshaw et al. (1974). Therefore, we have assumed that the nonrefractory state $M^{\ddagger}\cdot D\cdot P_i$ is a branch from the myosin ATPase cycle rather than being an intermediate in the myosin cycle occurring between $M^{**}\cdot D\cdot P_i$ and $M^{*}\cdot D\cdot P_i$, but clearly more work will be necessary to determine if this is in fact the case. In this regard, it is also possible that $M^{*}\cdot T$ is a branch from the myosin ATPase cycle with a direct transition from $M\cdot T$ to $M^{**}\cdot D\cdot P_i$ occurring. If this happened then $M^{**}\cdot D\cdot P_i$ would be the key state shared by both the myosin and actomyosin ATPase cycles, and both $M^{*}\cdot T$ and $M^{\ddagger}\cdot D\cdot P_i$ would be branches related to the interaction of the S-1 with actin. Finally, it should be noted that our experimental data do not rule out the possibility that the transition from the refractory state to the nonrefractory state is identical with the initial phosphate burst, $M^{*}\cdot T \rightleftharpoons M^{**}\cdot D\cdot P_i$. However, this would require that the initial burst be slower than previously reported, since the transition from the refractory to the nonrefractory state is the rate-limiting step in the cycle.

Following the two-step rebinding of S-1 to actin in our cycle, actin induces product release from the S-1. Since even at high actin concentration, the rate of reassociation of the actin and S-1 equals the steady-state ATPase rate, it follows that the other steps in the cycle must be considerably faster than this rate-limiting step. Therefore, our data strongly imply that product release from the acto-S-1 must be at least five times faster than the rate of reassociation of the actin and S-1. Otherwise the overall steady state ATPase rate would not be within 20% of the rate of reassociation of the actin and S-1. Therefore, in contrast to the original Lymn–Taylor model, in our cycle product release is not the rate limiting step.

The implications of this type of cycle for possible cross-bridge mechanisms in vivo will be discussed in a separate publication (Eisenberg and Hill, in preparation), but certain general points are worth considering here. First, in contrast to the original Lymn–Taylor model, we find that the S-1 undergoes two, rather than one, conformational changes after dissociation from actin. The first of these conformational changes yields an intermediate that does not bind to actin at all, i.e., the refractory state. If our assumption that this first conformational change is due to the initial hydrolysis of the bound ATP, it means that rather than nucleotide hydrolysis yielding a state that can rebind to actin, it yields a state that does not bind to actin until it undergoes some further conformational change, i.e., transforms to the nonrefractory state. The nature of this second conformational change and how it is related to the state of the bound nucleotide remains unclear.

The second major point to note about this cycle is that it, in fact, consists of two separate processes, dissociation and reassociation, each of which itself goes to completion. Since actomyosin was first discovered, it has been observed that in the presence of ATP at low ionic strength both in vivo and in vitro actin and myosin appear to only interact partially. That is, there is clearly more interaction than occurs with completely dissociated actomyosin but less than what occurs in the absence of ATP in rigor (Szent-Gyorgyi, 1947; Maruyama and Gergely, 1962a,b; Eisenberg and Moos, 1967). Our data suggest that the explanation for this phenomenon is not that relatively weak binding of the actin and myosin occurs in the presence of ATP but rather that there is a balance between the irreversible dissociation and reassociation parts of the cycle. On this basis, the dependence of the S-1 ATPase on actin concentration is not a measure of the strength of binding of the nonrefractory state to actin, but rather is a measure of the dependence of the rate of rebinding on the actin concentration (see Scheme II). Likewise, the number of cross-bridges attached to actin in vivo will depend not only on the rate of attachment but also on the rate of detachment which will be quite different in vitro and in vivo. Clearly, in vivo it will depend on the relative positions of the cross-bridge and the actin filament and thus on the velocity of contraction. Therefore, our cycle suggests that it is a balance between steady-state association and dissociation process rather than the establishment of an equilibrium that determines the amount of actin-myosin interaction that is observed under various conditions.

The third point to be noted about this cycle is that by providing a biochemical mechanism for making the rate of attachment of the cross-bridge rate limiting, our cycle provides possible explanations for several of the problems left unexplained by the Lymn–Taylor model where product release was postulated to be rate limiting. At a high velocity of contraction, the myosin cross-bridge is of necessity attached to the actin for such a short time that it did not seem possible for product release to be the rate-limiting step in vivo. Furthermore, the advantages of a relatively low rate of attachment of the cross-bridge in explaining the leveling off of energy utilization at high velocity was demonstrated by the 1957 model of A. F. Huxley (1957; 1974). By having the transition from the refractory to the nonrefractory state govern the rate of attachment of the cross-bridge, the control over this important step in a contracting muscle is placed in the myosin molecule alone, and this provides a direct link between the myosin ATPase rate and the velocity of muscle contraction.

In conclusion, we have observed a single cycle of ATP hydrolysis by acto-S-1 and found that the rate around the cycle is equal to the steady-state ATPase not only at low but also at high actin concentration. The rate-limiting step in the cycle is the rebinding of S-1 to actin, a two-step process that at high actin concentration levels off at the rate of the first-order transition from the refractory to the nonrefractory state. Since we can now observe several of the steps in the single cycle of ATP hydrolysis, it will be of interest in the future to determine how various agents affect each of these steps rather than just the rate-limiting actin–S-1 reassociation step which is all that can be determined by steady-state measurements.

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