The Mechanism of the Skeletal Muscle Myosin ATPase

1. Identity of the Myosin Active Sites*

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In the present study, the question of whether the two myosin active sites are identical with respect to ATP binding and hydrolysis was reinvestigated. The stoichiometry of ATP binding to myosin, heavy meromyosin, and subfragment-1 was determined by measuring the fluorescence enhancement caused by the binding of MgATP. The amount of irreversible ATP binding and the magnitude of the initial ATP hydrolysis (initial P, burst) was determined by dequenching [1-32P]ATP hydrolysis with and without a cold ATP chase in a three-syringe quenched flow apparatus. The results show that, under a wide variety of experimental conditions: 1) the stoichiometry of ATP binding ranges from 0.8 to 1 mol of ATP/myosin active site, 2) 80 to 100% of this ATP binding is irreversible, 3) 70 to 90% of the irreversibly bound ATP is hydrolyzed in the initial P, burst, 4) the first order rate constant for the rate-limiting step in ATP hydrolysis by heavy meromyosin is equal to the steady state heavy meromyosin ATPase rate only if the latter is calculated on the basis of two active sites per heavy meromyosin molecule. It is concluded that the two active sites of myosin are identical with respect to ATP binding and hydrolysis.

The biochemical mechanism of the myosin ATPase has been the subject of intensive investigation for many years. Most workers now agree that several phenomena accompany the interaction of ATP with myosin. An enhancement in tryptophan fluorescence (2, 3) and UV absorbance (4) occurs, H+ is released (5-8), and at least 0.5 mol of ATP/mol of active site is rapidly hydrolyzed in what has been called the initial P, burst (9-12). The pre-steady state kinetic studies of Tonomura (10, 11, 13), Trentham (3), and Taylor (8, 12) and their collaborators (10, 20) have suggested that the two myosin heads follow quite different mechanisms even when they have been separated into individual heads or subfragment-1 molecules. One head hydrolyzes ATP essentially by the mechanism given above while the second head hydrolyzes ATP by a quite different mechanism:

\[
M + ATP \xrightarrow{k_1} M \cdot T \xrightarrow{k_2} M^* \cdot D \cdot P, \quad \text{and} \quad M^* \cdot D \cdot P \xrightarrow{k_3} M + D + P, \quad (2)
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Here the binding of ATP, although relatively strong, is not irreversible but is in rapid equilibrium because in contrast to Scheme 1, \( k_{-1} \) is faster than the rate-limiting step \( k_2 \). Furthermore, the ATP is not rapidly hydrolyzed in the initial P, burst, but is slowly hydrolyzed in the rate-limiting step of the scheme.

In the present study, using both stopped flow and quenched flow techniques, we have reinvestigated whether the two myosin heads are identical with respect to the binding and hydrolysis of ATP. Because of the controversy surrounding this question and the difficulty of the measurements, we studied each of the steps in the myosin ATPase mechanism in turn rather than just the magnitude of the initial P, burst. The results show that, under a wide variety of conditions, both myosin heads bind ATP irreversibly and 75 to 90% of the irreversibly bound ATP is hydrolyzed in the initial P, burst. Furthermore, both myosin heads hydrolyze ATP at the same steady state rate. We, therefore, conclude that the two

\[
M + ATP \xrightarrow{k_1} M \cdot T \xrightarrow{k_2} M^* \cdot D \cdot P, \quad \text{and} \quad M^* \cdot D \cdot P \xrightarrow{k_3} M + D + P, \quad (1)
\]
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MATERIALS AND METHODS

Proteins—Myosin was purified from rabbit back and leg muscles according to the method of Kielley and Harrington (23). Heavy meromyosin was prepared from myosin by tryptic digestion as previously described (24). S-1 was prepared from myosin using soluble papain digestion as described by Lowey et al. (25). Protein concentrations were measured spectrophotometrically with extinction coefficients for a 1 mg/ml solution at 280 nm of 0.56, 0.647, and 0.77 cm⁻¹ for myosin, HMM, and S-1, respectively (23, 26). Molecular weights for myosin, HMM, and S-1 were taken as 480,000, 350,000, and 120,000, respectively.

Stopped Flow Experiments—The stopped flow experiments were performed using a custom-designed apparatus equipped with a temperature control capability (27, 29). Since the pre-steady state myosin ATPase reaction is extremely sensitive to temperature, to assure accurate temperature control, the room temperature was also cooled down close to the reaction temperature during the performance of the experiment. All of the fluorescence measurements were performed at an excitation wavelength of 360 nm obtained with a Bausch and Lomb monochromater and an emission wavelength of 430 nm (correction interference filter) detected with an EMI 9558QB-20 photomultiplier perpendicular to the incident light. The signals detected from the reaction chamber reaction change were digitized and processed in a DEC-PDP-11/05 computer. The first order waveforms were analyzed according to the equation ln (P₄₂ — P₄₃) = -kt where P₄₂ and P₄₃ were the relative millivolt readings at time t and at the end of the reaction, respectively. The pseudo-first order rate constant, k, was then obtained from the average slope of a plot of the ln (P₄₂ — P₄₃) versus t. When two or more waveforms of the same time course were compared, each waveform was first normalized according to the relationship (ln (P₄₂ — P₄₃)/P₄₂) where P₄₂ was the relative millivolt reading at the beginning of the reaction. The rate constant was then obtained from the average slope of a plot of the ln ((P₄₂ — P₄₃)/P₄₂) versus t. An example of this is shown in Fig. 1 where three separate reaction waveform are compared. The points on the curve are values obtained from each individual reaction waveform. The same procedure was also applied to the results obtained from the three-syringe quenched flow experiment whenever comparisons of reaction time courses are required, e.g. Fig. 4.

Quenched Flow Experiments—The quenched flow experiments were performed in a temperature-controlled Durrum D-132 three-syringe, quenched flow apparatus operated in the continuous flow mode. Each reaction time point represents the aging time of the reaction, i.e. the length of time between the mixing of the first two solutions and the quenching of the reaction. Variation of the length of time before the quench solution was added was achieved by varying the flow rate of the solution. The flow rate was monitored for each reaction by a calibrated linear velocity transducer, the output of which was displayed on a Tektronix storage oscilloscope. The variation in flow rate was achieved by varying both the pressure of the pneumatic system and the distance of the syringe tip from the purge and collect outlet ports. Teflon tubing of varying length and inside diameter (0.016 to 0.051 inches) was used. In this way, reaction time ranging from about 13 to 400 ms could be obtained. To prevent contamination by reaction solutions remaining in the system from the previous reaction, the first sample of each series of reactions was always discarded and the purge volume was always set at 4 to 7 times the dead volume of the reaction chamber. In a typical reaction, a volume of about 0.8 ml is ejected through the purge and then about 0.7 ml is collected through the collection port into a tared test tube. By rapid weighing, the sample size was made exactly 0.5 ml and it was then analyzed for [γ-32P]P. For measurement of irreversible ATP binding, the S-1 was first mixed with about a 5-fold molar excess of [γ-32P]ATP with a specific activity of about 5000 cpm/micromol of ATP and then quenched with a 100-fold molar excess of nonradioactive ATP from the third syringe. This sample was further incubated for about five half-lives of the steady state ATPase rate, i.e. about 2 to 4 min (depending on the reaction condition) before analysis of [γ-32P]ATP was carried out. In studies of the P₁ initial burst, the cold ATP quench solution was replaced with a 2 N HCl solution. To avoid hydrolysis of [γ-32P]ATP by the added acid the 0.5 ml sample was extracted for P₁ within 1 min after the sample was collected. The extraction procedure for P₁, was based on a modified method of Mulhern and Riesenberg (29). The 0.5-ml sample was made 1 M in HCl by adding either 0.1 ml of 6 N HCl to samples quenched with cold ATP or 0.1 ml of 1 N HCl to samples quenched with 2 N HCl. To the resulting 0.6 ml samples, 0.25 ml of 5 M H₂SO₄, 6% silicotungstic acid was then added. Addition of both HCl and H₂SO₄/silicotungstic acid was found to be necessary to completely extract the P₁ into the organic phase since myosin and its fragments tended to inhibit complete extraction of the P₁. To facilitate extraction of the P₁, 1 mm cold P₁, was always present as carrier. After addition of the H₂SO₄/silicotungstic acid, the solution was blended briefly on a Vortex mixer and then 1 ml of a 1:1 isobutyl alcohol/benzene solution and 0.25 ml of 5% ammonium molybdate solution were added. After blending on a Vortex mixer for 30 s to extract the [γ-32P]P into the organic phase, a 0.25-ml aliquot of the organic phase was transferred into a scintillation vial and counted for [32P], after addition of 15 ml of Aquasol.

[γ-32P]ATP was purchased from New England Nuclear. Determination of the total amount of counts present as [γ-32P]ATP was performed by completely hydrolyzing a given sample with S-1. In general, about 90% of the counts were present as [γ-32P]ATP. The remaining 10% were nonhydrolyzable contaminants rather than [γ-32P]ATP. Infrared absorption bands rather than [γ-32P]ATP. Blank measurements were performed in the three-syringe stopped flow apparatus in a manner identical to the actual experiments except the enzyme was replaced with buffer. In these blank experiments no more than 2% of the [γ-32P]ATP added was present as [32P]P, at the end of the procedure. For determination of the rates and magnitudes of the irreversible ATP binding and the initial P₁ burst, generally a 4-fold molar excess of ATP over enzyme active sites was added. Therefore the blank correction was no more than 8% for a reaction which went to completion (all of the bound ATP hydrolyzed) and no more than 40% even for the first time point in a rate measurement, when only about 20% of the bound ATP was hydrolyzed.

RESULTS

In this study of the binding and hydrolysis of ATP, we initially used fluorescence enhancement as a measure of ATP binding. Werber et al. previously showed that a fluorescence enhancement occurs when ATP binds to myosin (2). Fig. 1 shows the time course of the fluorescence enhancement which occurs when ATP binds to myosin, HMM, or S-1. As can be seen, for all three proteins, the rate of ATP binding can be fitted by a single exponential. There is no evidence that ATP binds to the two myosin heads at markedly different rates. Furthermore, this is not because only one of the two heads is binding ATP. As shown in Fig. 2, when the magnitude of the fluorescence change is plotted as a function of ATP concentration, the fluorescence magnitude levels off when 1 mol of ATP binds/mol of myosin head. Therefore both myosin heads appear to bind ATP quite tightly.

The simplest interpretation of the data in Figs. 1 and 2 is that both myosin heads bind ATP at the same rate and the binding of ATP to both heads causes an increase in fluorescence. However, an alternative explanation is possible. The binding of ATP to one of the myosin heads in accordance with Scheme 1 might cause an increase in fluorescence, while the binding of ATP to the second head in accordance with Scheme 2 might not be irreversible or cause a change in fluorescence, but it could still be quite strong. Thus, if the binding constant of ATP to myosin in Scheme 2 were about 10⁻⁶ M⁻¹, the titration plot in Fig. 2 would still level off at 1 mol of ATP/mol of active site even if one of the myosin heads did not contribute to the fluorescence enhancement. Therefore, the fluorescence data cannot rule out that both myosin heads bind ATP strongly but with different mechanisms as suggested by Tomonura and his collaborators (10, 20). What is required is a direct determination of the amount of ATP bound irreversibly and the amount rapidly hydrolyzed in the initial P₁ burst.

Using a three-syringe quenched flow apparatus, the amount
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Fig. 1. Kinetics of the fluorescence enhancement of myosin, HMM, and S-1 by ATP. Conditions: 100 µM ATP, 0.5 M KCl, 9 mM MgCl2, 20 mM Tris-HCl, pH 7.9, 20°C. A, time course of the normalized fluorescence change following the addition of 100 µM ATP to 4 µM myosin heads (○), 5.8 µM HMM heads (△), and 5.3 µM S-1 (●). The points on the curve are normalized values obtained from separate oscilloscope traces of the experiments performed on the respective proteins. B, first order log plot of the data in A. The observed rate constant obtained is 45 s⁻¹ for all three enzymes.

Fig. 2. Active site titration of myosin, HMM, and S-1. Conditions same as Fig. 1. ΔF is the relative amplitude of fluorescence enhancement at a given ATP, ΔF_max is the maximum amplitude of fluorescence enhancement at high ATP, e.g., 50 µM or more. ○, 4.5 µM myosin heads; △, 5.8 µM HMM heads; ●, 5.3 µM S-1. ---, extrapolation of the titration curve, showing that the maximum level of fluorescence enhancement occurs when each myosin site binds one ATP.

Of ATP bound irreversibly to the myosin can be determined by mixing the enzyme with [γ-32P]ATP followed by addition of a large excess of nonradioactive ATP within 10 to 300 ms, i.e., by a cold ATP chase. Any [γ-32P]ATP which is reversibly bound will dissociate from the myosin and be diluted out by the nonradioactive ATP without being hydrolyzed. In contrast, the irreversibly bound [γ-32P]ATP will all be hydrolyzed and the radioactive P_i will be released at the steady state ATPase rate. Therefore, determination of the amount of [γ-32P]ATP, formation a few minutes after the cold ATP chase will yield a value for the amount of ATP irreversibly bound. The amount of ATP hydrolyzed in the initial P_i burst can be determined by mixing the enzyme with [γ-32P]ATP followed directly by an acid quench which will denature the myosin and release any bound [γ-32P]ATP.

To determine the maximum amount of irreversibly bound ATP and the maximum amount of the P_i burst, the rates of the ATP binding and P_i burst must first be determined to be certain that the ATP binding and P_i burst have reached their maximum value. Furthermore, it is important to calibrate the three-syringe, quenched flow apparatus. This can be done by comparing, at low ATP concentration, the rate of the fluorescence enhancement measured in the two-syringe, stopped flow apparatus with the rates of the irreversible ATP binding and the initial P_i burst measured in the three-syringe, quenched flow apparatus.

Although both myosin heads may not bind ATP irreversibly, the heads which show the fluorescence change presumably also bind ATP irreversibly and show the initial P_i burst, i.e., they follow Scheme 1. Scheme 1 predicts that, under conditions where the binding of ATP is, itself, rate-limiting, the rates of the fluorescence enhancement (M*T or M**D·P_i formation, or both), the irreversible ATP binding (M*T formation), and the initial P_i burst (M**D·P_i formation) will all be equal to the rate of ATP binding. Fig. 3 shows the rate observed for the fluorescence enhancement of S-1 as a function of ATP concentration at 0.5 M KCl. Under this condition, the rate constant for the fluorescence enhancement doubles each time the ATP concentration doubles. Therefore, under this condition the binding of ATP is rate-limiting for all of the heads which follow Scheme 1. Hence, at the ATP concentration shown by the arrow in Fig. 3, the rate of the irreversible ATP binding, the rate of the initial P_i burst, and the rate of the fluorescence change should all be equal. The data in Fig. 4 show that this is indeed the case. The rates of all three phenomena can be fitted by a single exponential.
Magnitude of the irreversibly bound ATP and the initial P, burst

The data were obtained at 0.5 M KCl, 5 mM MgCl, 20 mM Tris buffer, pH 7.9, 15°C with 12.5 μM active site concentration and 100 μM [γ-32P]ATP. 15 mM unlabeled ATP was used for the quenching solution for the determination of the irreversible ATP binding, while 2 N HCl was used for the quenching solution for the determination of the initial P, burst. The incubation time for the reaction was 350 ms. The irreversibly bound ATP includes M* -T and M** -D-P, while the initial P, burst measures M** -D-P.

This shows that the three-syringe, quench-flow apparatus is operating correctly. Furthermore, the pseudo-first order rate constant is 18 s⁻¹ so that by 250 ms all three phenomena are essentially complete. Therefore, under this condition, the maximum amount of ATP irreversibly bound to S-1 or hydrolyzed by S-1 in the initial P, burst can be determined at 250 ms.

Table I shows these data for myosin, HMM, and S-1. For all three proteins, the amount of ATP irreversibly bound and the amount of ATP hydrolyzed in the initial P, burst is close to 1 mol/mol of S-1. These data are not consistent with a model where only half of the myosin heads bind ATP irreversibly and hydrolyze it in the initial P, burst.

Under the conditions of Fig. 4, the amount of irreversibly bound ATP and the magnitude of the initial P, burst determined at 250 and 400 ms after mixing are very close to their maximal value. In fact, under most conditions, 250 or 400 ms are convenient times to determine the amount of irreversibly bound ATP and the magnitude of the initial P, burst. Any reaction with a rate constant of 10 s⁻¹ or greater will be more than 98% complete at 400 ms. On the other hand, at 400 ms even if the steady state ATPase is as high as 0.1 s⁻¹, the correction of the initial P, burst for ATP hydrolyzed during steady state hydrolysis will be less than 5%. Therefore, we determined the amount of irreversibly bound ATP and the magnitude of the initial P, burst at 250 or 400 ms, or both, for myosin, HMM, and S-1 under a variety of conditions. Table II shows that, under all conditions tested, the magnitude of the irreversibly bound ATP is about 0.8 mol/mol of myosin head. At 0.5 M KCl, almost all of the ATP which is irreversibly bound to the myosin heads is hydrolyzed in the initial P, burst so that the magnitude of the initial P, burst is also close to 0.8 mol/mol of myosin head.

At lower salt concentrations, the magnitude of the initial P, burst is somewhat lower than the amount of irreversibly bound ATP, possibly because the equilibrium between M* -T and M** -D-P shifts toward M* -T to some extent (12). Of course, it is also possible that, at very low salt concentration, about 25% of the S-1 does not show the initial P, burst, this would have to be some form of partial denaturation. In this regard, it has been reported that certain preparations of cardiac myosin which still hydrolyze ATP show a very low magnitude for the initial P, burst (30). Experiments determining the amount of 18O exchange occurring on bound ATP (31) may help determine whether relatively low values for the magnitude of the initial P, burst are due to an equilibrium between M* -T and M** -D-P, or to partial denaturation of the S-1. In any event, taken as a whole, the data in Tables I and II strongly imply that, under a wide variety of conditions, both myosin heads bind ATP irreversibly and are able to hydrolyze it rapidly in the initial P, burst.

Thus far in this paper, our experiments have indicated that both myosin heads bind ATP irreversibly at the same rate and hydrolyze ATP in the initial P, burst at the same rate.
The next step in the myosin ATPase mechanism is the slow rate-limiting transition from $M^{**}\cdot D\cdot P$ to $M^*\cdot D\cdot P$. We therefore investigated whether this step has the same rate for both myosin heads. If, in a stopped flow experiment, the concentration of ATP mixed with HMM is equal to or less than the concentration of HMM heads, then, following the initial fluorescent enhancement observed in Fig. 1, a much slower fluorescence decay is observed. As can be seen in Fig. 5, with HMM at 0.1 mM KCl, pH 8, 20°C, this fluorescence decay fits a simple exponential with a rate constant of 0.032 s$^{-1}$. There is no evidence that the rate of fluorescence decay is biphasic, as might be expected if the rate-limiting step were different for the two myosin heads.

Further evidence that both heads hydrolyze ATP at the same rate comes from a comparison of the rate of fluorescence decay and the steady state ATPase rate. Based on Scheme 1, the rate constant for fluorescence decay equals $k_4\frac{M^{**}\cdot D\cdot Pi}{M_{total}}$, i.e. $k_4k_{b1}/(k_3 + k_{-3})$ (3). The rate constant for the steady state ATPase will also equal $k_4k_{b1}/(k_3 + k_{-3})$ if both HMM heads hydrolyze ATP at the same rate. The steady state ATPase rate can be measured directly on the stopped flow apparatus. This rate is calculated from the time required to hydrolyze all of the ATP added in excess of the HMM, i.e. the length of time between the initial rise in fluorescence which occurs when the ATP and HMM are first mixed and the decay in fluorescence which occurs when the free ATP is all hydrolyzed. Fig. 6 shows such an experiment under the same conditions as Fig. 5 and the inset depicts the time required to hydrolyze the added ATP as a function of ATP concentration. From the slope, a value of 0.03 s$^{-1}$ was calculated for the steady state ATPase rate constant with the assumption that two active sites are present per HMM molecule. This value for the steady state ATPase rate constant is similar to the value for the steady state ATPase rate constant we obtain using the conventional pH-stat method. The agreement between the value for the rate constant calculated on the basis of two active sites per HMM molecule (Fig. 6) and the rate constant obtained for the fluorescence decay (Fig. 5) strongly suggests that both myosin heads hydrolyze ATP at the same steady state rate.

**Fig. 5.** Fluorescence change during hydrolysis of 1 ATP molecule/HMM head. Conditions. 10 pM HMM heads, 0.1 mM KCl, 0.5 mM MgCl$_2$, 20 mM Tris-HCl, pH 8, 20°C. The reaction was initiated by adding 1 mol of ATP/mol of HMM head (10 pM). Curve a, time course of the fluorescence decay which immediately follows the fast initial enhancement (not shown); Curve b, computer-generated first order log plot of a which yields a first order rate constant of 0.032 s$^{-1}$.

**Fig. 6.** Steady state HMM ATPase determined on the stopped flow apparatus. Conditions same as Fig. 5. Inset, time course of the fluorescence change following the fast initial fluorescence enhancement (not shown) when 100 pM ATP is mixed with 10 pM HMM sites in the stopped flow apparatus; arrow, time when the fluorescence has decayed to half its amplitude. The length of time (t) it takes for the reaction to reach the designated point is proportional to the concentration of ATP added. The plot in the figure shows the dependence of t on ATP concentration. From the slope of this plot, a steady state ATPase rate constant of 0.03 s$^{-1}$ is obtained for the experimental condition employed in Fig. 5.

**DISCUSSION**

One of the major controversies concerning the myosin ATPase is whether both heads of the myosin molecule hydrolyze ATP by the same mechanism. Tonomura and his collaborators have argued that the mechanism followed by the two heads is different (10, 20). Moreover, they suggest that this difference is not dependent on a cooperative interaction between the two myosin heads. Rather, the two heads are reported to have different properties even when separated as individual S-1 molecules (22, 32, 33). In the present study, we approached this problem by investigating all of the steps which the myosin molecule undergoes as it interacts with ATP. We studied the binding of ATP both by measuring the accompanying fluorescence change and by directly measuring the amount of irreversibly bound ATP. We also investigated the magnitude of the rapid ATP hydrolysis during the initial Pi burst. Finally, we investigated the rate of the fluorescence decay and compared it with the steady state ATPase rate. All of these measurements suggest that both of the myosin heads act identically in regard to the binding and hydrolysis of ATP. These results agree with the studies of Trentham (14), Taylor (11, 12), Weeds (19), and their colleagues but disagree with the studies of Tonomura (10, 20) and Tawada (21, 22) and their colleagues.

Could this disagreement be due to an error in our measurements? One of the reasons we studied a number of different aspects of the myosin-ATP interaction was to avoid the possibility that an artifact in a particular measurement would lead to an erroneous conclusion. Our fluorescence titration data are in agreement with numerous other studies which show that both myosin heads bind ATP quite tightly. Furthermore, the rate of ATP binding, as measured by the rate of the fluorescence change, shows no evidence of being different for the two myosin heads. However, the strongest evidence that the two myosin heads are identical comes from our data on the irreversible binding of $\gamma^{32}$P$^\alpha$ATP. One of the major assumptions of the Tonomura scheme is that one of the two myosin heads binds ATP in a rapidly reversible equilibrium followed by a much slower hydrolysis step. If this, in fact, does occur then, when $[\gamma^{32}$P$]ATP is rapidly mixed with myosin followed by a cold ATP chase, the $[\gamma^{32}$P$]ATP should remain bound to only half of the myosin heads. On the other heads, the $[\gamma^{32}$P$]ATP should be displaced by the cold ATP long before it is hydrolyzed so that no more than half the myosin heads bind ATP irreversibly.
In agreement with the results of Taylor (12) and Bagshaw and Tretham (14), our results strongly suggest that both myosin heads bind ATP irreversibly. Using myosin, HMM, or S-1 under a wide variety of conditions, we find that the amount of irreversibly bound ATP ranges from 80 to 100%. It is difficult to see how these data can be consistent with a model where only one of the two myosin heads binds ATP irreversibly. Kanazawa and Tonomura have argued that measurements of the initial Pi burst could be erroneously high due to "extra" ATP hydrolysis (9), but this should not affect determination of the amount of irreversibly bound ATP. Generally, 10 to 20% of the heads do not bind ATP irreversibly, quite possibly because this small number of myosin heads are denatured (34). However, with none of our preparations have we ever observed only 50% of the myosin heads binding ATP irreversibly as predicted by the Tonomura model (20).

Although these results strongly suggested that both myosin heads bound ATP irreversibly, it still remained possible that only one of the two myosin heads hydrolyzed ATP in the initial Pi burst. We therefore measured the magnitude of the initial Pi burst. However, we made no attempt to make this measurement by extrapolation of the steady state ATPase rate, the method employed by Tonomura and his collaborators (10, 20). Even if both myosin heads are identical, the magnitude of the P, burst would not be expected to exceed 70 to 80% of the total number of myosin heads present due both to slight denaturation and to an equilibrium between M* . T and M** . D . P,. Therefore, small errors in extrapolation of the steady state ATPase rate could make the magnitude of the initial P, burst appear to be only 50 to 60% of the total number of myosin heads present. To avoid these errors in extrapolation, we measured the magnitude of the initial P, burst at 250 or 400 ms where P1 production due to steady state hydrolysis is negligible while at a first order reaction with a rate constant of 10 s^-1 or greater is nearly complete. Our results strongly suggest that more than half of the myosin heads show the initial P, burst. At 0.5 M KCI, where the equilibrium between M* . T and M** . D . P, is strongly shifted toward M** . D . P, the magnitude of the initial P, burst is about 0.8/mol of myosin head. In making these P, measurements, we found that the presence of relatively high myosin concentrations prevented complete extraction of the phosphomolybdate into the isobutyl alcohol/benzene phase leading to erroneously low estimates of P, present. The use of both HCl and a mixture of silicotungstic/sulfuric acid as quenching agents (see "Materials and Methods") helped to resolve this difficulty. Whether this problem plays any role in the low values for the magnitude of the P1, burst observed by Tononura and his collaborators is unknown. Another possible explanation for their low values is that their protein preparations are partially denatured. This possibility is particularly strong for S-l which does not show the initial P, burst and have found that this fraction also shows a very low calcium, EDTA, and actin-activated ATPase activity (33). In any event, although we cannot rule out that a small fraction of the S-l in our experiments is partially denatured and does not show the initial P, burst, we conclude on the basis of our measurements that, in general, both myosin heads hydrolyze ATP in the initial P, burst.

To complete our investigation of the identity of the two myosin heads, we investigated the overall rate of ATP hydrolysis by HMM. Based on the model given in Scheme 1, at saturating ATP concentration, assuming k - is negligible, the steady state ATPase rate should equal k-k1/(k3 + k-), i.e. (M**.D.P./M_total)k1. This should also be the rate constant for the fluorescence decay, assuming that the mixture of M* . D . P, and M have a lower fluorescence than the mixture of M* . T and M** . D . P.. The rate of fluorescence decay yields a first order rate constant which is independent of any assumption of molecular weight for the active site. On the other hand, to obtain a first order rate constant from the steady state ATPase data, an assumption must be made about the number of active sites present which hydrolyze ATP. If we assume that there are two active sites present per HMM molecule, the calculated rate constant for the steady state ATPase is equal to the measured rate constant for the fluorescence decay as predicted by Scheme 1. On the other hand, if only one active site was hydrolyzing ATP per HMM molecule but at twice the rate, the calculated steady state ATPase rate constant would not change but the rate constant for the fluorescence decay of this site would be double the rate constant we actually observe. Therefore, our data suggest that both HMM heads hydrolyze ATP at the same rate.

It should be noted that, although these data imply that both HMM heads hydrolyze ATP, they do not eliminate the possibility that the two heads operate in a highly cooperative manner, i.e. one myosin head never hydrolyzes an ATP molecule unless the other myosin head hydrolyzes ATP at the same time. If such extreme cooperative behavior occurred, both the steady state ATPase rate constant and the rate constant for fluorescence decay would be twice as fast for HMM as for S-l. However, due to the possibility that small differences between HMM and S-1 could be caused by differences in proteolytic digestion, it is difficult to make meaningful comparisons of the steady state ATPase rates of HMM and S-1.

Summarizing the data presented in this paper, then, the results indicate that both myosin heads bind ATP irreversibly and rapidly hydrolyze it in the initial P, burst. No evidence is found for a significant population of myosin heads which bind ATP reversibly and show no initial P, burst.

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