The Mechanism of the Skeletal Muscle Myosin ATPase

II. RELATIONSHIP BETWEEN THE FLUORESCENCE ENHANCEMENT INDUCED BY ATP AND THE INITIAL P. BURST*

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A major question about the mechanism of the myosin ATPase is how much of the fluorescence change which accompanies the binding of ATP to myosin is due to the conformational change induced by ATP and how much is due to the subsequent hydrolysis of ATP in the initial P concentration; 3) the rate of the irreversible ATP binding, the rate of the initial P burst, and the rate of the fluorescence enhancement were compared under varied conditions. The results show that: 1) the fluorescence enhancement is mainly due to the hydrolysis of ATP in the initial P burst rather than to the conformational change induced by ATP; 2) the rate of the initial P burst is considerably slower than the rate of irreversible ATP binding at high ATP concentration; 3) the rate of the initial P burst is qualitatively the same as the rate of the fluorescence enhancement. Therefore, the maximum rate of the fluorescence enhancement represents the rate of the initial P burst rather than the rate of the conformational change induced by ATP binding.

In the accompanying paper (3), we presented evidence, in agreement with the work of Taylor (4, 5), Trentham (6), Weeds (7) and their collaborators, that both heads of the myosin molecule follow the same biochemical mechanism in their interaction with ATP. Both heads bind ATP irreversibly and then rapidly hydrolyze it in the initial P burst. However, certain important details of this mechanism remain unclear. Using the formalism of Bagshaw et al. (8), where M is myosin, T is ATP, D is ADP, Pi is inorganic phosphate, and * and ** qualitatively represent the amount of fluorescence enhancement shown by the various intermediates, the basic mechanism can be written as follows:

\[
M + T \rightarrow M \cdot T \rightarrow M^* \cdot T \rightarrow M^{**} \cdot D \cdot P; \quad M^{**} \cdot D \cdot P \rightarrow M + D + P,
\]

One of the key features of this model is that the binding of ATP is postulated to occur in two steps: weak binding of ATP in the collision intermediate (M \cdot T), followed by an irreversible conformational change to form M^* \cdot T. The major evidence for such a two-step binding process is that at 0.1 M ionic strength, the rate of the fluorescence enhancement which accompanies ATP binding, levels off at high ATP concentration (8). If the binding of ATP were a single step process, the rate of the fluorescence enhancement would be directly proportional to the ATP concentration used. Since the rate of the fluorescence enhancement as well as the rate of H' release level off at high ATP concentration, both Bagshaw et al. (8) and Koretz and Taylor (9) have suggested that these phenomena are caused by the conformational change, M \cdot T \rightarrow M^* \cdot T. However, it is not clear whether all of the fluorescence enhancement is associated with the binding of ATP or whether the subsequent hydrolysis of ATP in the initial P burst affects both the magnitude and the rate of the fluorescence change. Werber et al. (10) suggested that the fluorescence enhancement was mainly due to the presence of the intermediate, M^{**} \cdot D \cdot P. Bagshaw et al. (8) and Bagshaw and Trentham (11) suggested on the basis of indirect evidence from analogue studies that approximately half of the fluorescence enhancement observed with ATP was due to M^* \cdot T and half to M^{**} \cdot D \cdot P. However, they assumed that the transition from M^* \cdot T to M^{**} \cdot D \cdot P, i.e. the initial P burst, was faster than the conformational change from M \cdot T to M^* \cdot T so that only the rate of the latter conformational change was detected in studies on the fluorescence enhancement. In contrast, Taylor (5) and Sleep and Taylor (12) reported that the rate of the initial P burst was slower than the rate of the ATP binding, i.e. the rate of the transition from M \cdot T to M^* \cdot T. However, because the rate of the fluorescence enhancement was equal to the rate of ATP binding and showed no evidence of being biphasic, Taylor (5) and Sleep and Taylor (12) suggested that all of the fluorescence enhancement was due to the transition from M \cdot T to M^* \cdot T with no fluorescence change accompanying the subsequent initial P burst. More recently, however, Johnson et al. (13) also suggested that part of the fluorescence change is due to the initial P burst.

In the present study, we investigated the rate of the fluorescence enhancement, the rate of irreversible ATP binding, and the rate of the initial P burst to determine if the model of Bagshaw et al. (8) for the myosin ATPase is correct. The results show that, in fact, the fluorescence enhancement is due mainly to the hydrolysis of ATP in the initial P burst rather than to the binding of ATP. Furthermore, because the rate of the hydrolysis step is slower than the rate of ATP binding at high ATP concentration, the data strongly suggest that the maximum rate of the fluorescence enhancement represents the rate of the initial P burst rather than the rate of the conformational change induced by ATP binding.

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MATERIALS AND METHODS

Myosin, heavy meromyosin, and subfragment-1 were prepared according to the methods described in the accompanying paper (3). The [γ-32P]ATP was purchased from either ICN Pharmaceutical or New England Nuclear with specific activity ranging from 5 to 25 Ci/mmol and was diluted with unlabeled ATP to a specific activity of about 8000 cpm/μmol of ATP. ATP was purchased from Sigma and all other reagents were of reagent grade.

The fluorescence experiments were performed in the two-syringe, stopped flow apparatus while the initial P, burst and the irreversible ATP binding experiments were performed using the Durrum D-132 three-syringe, quenched flow apparatus as described in the accompanying paper (3). The excitation wavelength was 300 nm and the fluorescence emission was monitored using a Corion interference filter with peak transmission at 340 nm. The concentrations given in each figure represent the final reaction chamber concentrations for the reaction, and all stopped flow time courses represent a computer average of four different waveforms obtained from four consecutive samplings of the same reaction. For the quenched flow experiments, the time course was constructed from the average values of duplicate samples for each time point. The observed rate constant for each reaction waveform is obtained according to the method described previously (14). When rates of different experiments are compared, the time course of each experiment is normalized according to the relationship: (P, - P0)/(P, - P0) where P0, P, and P are the concentrations at the beginning of the reaction, the end of the reaction, respectively (3).

RESULTS

Fig. 1A shows, for S-1 (first part of Scheme 1):  
\[ M + T \xrightarrow{k_1} M \cdot T \xrightarrow{k_2} M^* \cdot T \]  
(2)

The fluorescence change was then assumed to be due to the formation of both \( M^* \cdot T \) and \( M^{**} \cdot D-P_\text{n} \). (see Scheme 1) but since the rate of the formation of \( M^{**} \cdot D-P_\text{n} \), \( k_3 + k_{3a} \), in Scheme 1, was assumed to be much faster than \( k_2 \), the rate of the fluorescence change here depended only on \( k_1 \) and \( k_5 \). As shown by Bagshaw et al. (8), on this basis, under pseudo-first order conditions such that \( [T] \gg [M] \), the value of \( k_{obs} \), derived with the assumption \( k_{-1} \gg k_2 \), can be expressed as:  
\[ k_{obs} = \frac{k_2}{1 + \frac{k_1}{k_2[T]}} \]  
(3)

Since the binding of ATP is essentially irreversible, i.e. the ordinate intercept in Fig. 1A is approximately 0, \( k_{-2} \) is negligible. With this modification, Equation 3 predicts that a double reciprocal plot of \( 1/k_{obs} \) versus \( 1/[T] \) will yield a straight line with \( 1/k_2 \) as the ordinate intercept and \(-1/k_{2-1} \) as the abscissa intercept (Fig. 1B). However, as discussed by Bagshaw et al. (8), the data in Fig. 1A can also be interpreted by a different two-step mechanism which involves a myosin isomerization step prior to ATP binding, i.e.  
\[ M \xrightarrow{k_1} M^* \xrightarrow{k_2} M^* \cdot T \]  
(4)

On the basis of this mechanism, the pseudo-first order rate constant, \( k_{obs} \), derived with the assumptions that \( k_{-1} \gg k_1 \) and \( k_{-2} \approx 0 \) is  
\[ k_{obs} = \frac{k_1}{1 + \frac{k_{-1}}{k_2[T]}} \]  
(5)

Equation 5 indicates that a plot of \( 1/k_{obs} \) versus \( 1/[T] \) should also yield a straight line with \( 1/k_1 \) as the ordinate intercept and \(-1/k_{2-1} \) as the abscissa intercept.

To differentiate these two mechanisms, the rate of the fluorescence enhancement was determined as a function of HMM concentration under conditions where the added HMM concentration was much greater than the added ATP concentration. If the mechanism of ATP binding follows Scheme 2, then a double reciprocal plot of \( 1/k_{obs} \) versus \( 1/[M] \) will be identical to the double reciprocal plot of \( 1/k_{obs} \) versus \( 1/[ATP] \) obtained where the added ATP concentration is much greater than the added HMM concentration. This is because in Scheme 2 the HMM and ATP are symmetric, i.e. changes in either species cause the same effect on the rate of the reaction. On the other hand, the integrated equation describing the time course for the consumption of ATP with \([HMM] \gg [ATP]\), contains an exponential term and a linear term. Since the observed time course followed a single exponential function, the linear term can be assumed to be constant, so that \( k_{obs} \) can be expressed as:  
\[ k_{obs} = \frac{k_1k_2[M]}{k_1 + k_{-1}} \]  
(6)

Equation 6 indicates that \( k_{obs} \) is a linear function of \([M]\). Therefore, a plot of \( 1/k_{obs} \) versus \( 1/[ATP] \) (open circles) or \( 1/[HMM] \) (solid circles). Similar results were obtained using S-1 (Fig. 2B).

Fig. 2A depicts a double reciprocal plot of \( 1/k_{obs} \) versus \( 1/[ATP] \) (open circles) or \( 1/[HMM] \) (solid circles). Since the binding of ATP is essentially irreversible, i.e. the ordinate intercept in Fig. 1A is approximately 0, \( k_{-2} \) is negligible. With this modification, Equation 3 predicts that a double reciprocal plot of \( 1/k_{obs} \) versus \( 1/[T] \) will yield a straight line with \( 1/k_2 \) as the ordinate intercept and \(-1/k_{2-1} \) as the abscissa intercept (Fig. 1B). However, as discussed by Bagshaw et al. (8), the data in Fig. 1A can also be interpreted by a different two-step mechanism which involves a myosin isomerization step prior to ATP binding, i.e.  
\[ M \xrightarrow{k_1} M^* \xrightarrow{k_2} M^* \cdot T \]  
(4)

On the basis of this mechanism, the pseudo-first order rate constant, \( k_{obs} \), derived with the assumptions that \( k_{-1} \gg k_1 \) and \( k_{-2} \approx 0 \) is  
\[ k_{obs} = \frac{k_1}{1 + \frac{k_{-1}}{k_2[T]}} \]  
(5)

Equation 5 indicates that a plot of \( 1/k_{obs} \) versus \( 1/[T] \) should also yield a straight line with \( 1/k_1 \) as the ordinate intercept and \(-1/k_{2-1} \) as the abscissa intercept.

To differentiate these two mechanisms, the rate of the fluorescence enhancement was determined as a function of HMM concentration under conditions where the added HMM concentration was much greater than the added ATP concentration. If the mechanism of ATP binding follows Scheme 2, then a double reciprocal plot of \( 1/k_{obs} \) versus \( 1/[M] \) will be identical to the double reciprocal plot of \( 1/k_{obs} \) versus \( 1/[ATP] \) obtained where the added ATP concentration is much greater than the added HMM concentration. This is because in Scheme 2 the HMM and ATP are symmetric, i.e. changes in either species cause the same effect on the rate of the reaction. On the other hand, the integrated equation describing the time course for the consumption of ATP with \([HMM] \gg [ATP]\), contains an exponential term and a linear term. Since the observed time course followed a single exponential function, the linear term can be assumed to be constant, so that \( k_{obs} \) can be expressed as:  
\[ k_{obs} = \frac{k_1k_2[M]}{k_1 + k_{-1}} \]  
(6)

Equation 6 indicates that \( k_{obs} \) is a linear function of \([M]\). Therefore, a plot of \( 1/k_{obs} \) versus \( 1/[ATP] \) (open circles) or \( 1/[HMM] \) (solid circles). Similar results were obtained using S-1 (Fig. 2B).

In all cases, the straight lines do not intersect the origin. Instead, as predicted by Scheme 2, the double reciprocal plots with either protein or ATP in excess are identical. These data exclude the mechanism (Scheme 4) involving a protein isomerization step prior to ATP binding. Hence, the fluorescence enhancement associated with ATP binding is due to a process which follows the binding of ATP.

We next investigated how much of the fluorescence enhancement is due to the conformational change which immediately follows ATP binding (\( M \cdot T \rightarrow M^* \cdot T \)), and how much to the subsequent hydrolytic step (\( M^* \cdot T \rightarrow M^{**} \cdot D-P_\text{n} \)). As was pointed out above, developing Scheme 1 for the mechanism of ATP binding, Bagshaw et al. (8) assumed that the rate constant for the initial P, burst was faster than the rate constant for the conformational change \( M \cdot T \rightarrow M^* \cdot T \). On this basis, they suggested that the rate of the fluorescence increase was unaffected by the rate of the initial P, burst. However, when we performed an experiment similar to the experiment shown in Fig. 1, but at very low ionic strength, the results were inconsistent with those expected for Scheme 2. As shown in Fig. 3A, the value of \( k_{obs} \) for the fluorescence enhancement does plateau at high ATP concentration as expected. However, the \( k_{obs} \) does not follow a hyperbolic function with respect to ATP concentration as is demonstrated by the nonlinear double reciprocal plot of these data shown in Fig. 3B. This experiment was repeated numerous times and at very low ionic strength the plot was always concave rather than linear. Since \( k_{obs} \) levels off at high ATP concentration the data in Fig. 3 suggest that a two-step process occurs when ATP binds to myosin. However, since the double reciprocal plot of \( k_{obs} \) versus ATP is not linear, the data in Fig. 3 also suggest that the first step in the binding of ATP is
Fluorescence Enhancement and the Initial Pi Burst of Myosin

Fig. 1. Dependence of the rate of the fluorescence enhancement on the ATP concentration. A, direct plot of the observed pseudo-first order rates of fluorescence enhancement induced by ATP; B, double reciprocal plot of the curve in A. The experiments were done by mixing 2 μM S-1 with varying concentrations of ATP (reaction chamber concentrations) in a two-syringe, stopped flow apparatus. The reaction medium contained 0.1 mM KCl, 9 mM MgCl₂, 20 mM Tris buffer, pH 7.9, 20°C.

Fig. 2. Comparison of the dependence of the rate of the fluorescence enhancement on varying ATP concentration and on varying enzyme concentration. A, enzyme is HMM; B, enzyme is S-1. ○, experiments done under pseudo-first order conditions with respect to HMM or S-1 sites, i.e. the concentration of HMM or S-1 sites is kept at least 5 times greater than the ATP concentration; ◊, experiments done under pseudo-first order conditions with respect to ATP, i.e. the ATP concentration is at least 5 times greater than the HMM or S-1 site concentration. Conditions for both A and B: 40 mM KCl, 1.5 mM MgCl₂, 10 mM imidazole buffer, pH 7, 5°C.

not in rapid equilibrium as proposed in Scheme 2. This, in turn, suggests that the two steps might be first, an essentially irreversible binding of ATP which causes a relatively small increase in fluorescence and then the initial Pi burst which is responsible for the major part of the fluorescence enhancement:

\[
M + T \xrightarrow{K_1} M^* \cdot T \xrightarrow{k_2} M^{**} \cdot D \cdot P,
\]

where \( K_1 \) is the equilibrium constant for the formation of the collision intermediate, \( M \cdot T \). Although in this scheme we assume that the collision intermediate \( M^* \cdot T \) forms prior to \( M^{**} \cdot T \) formation, we also assume that \( k_2 \gg k_3 \) so that the rate of formation of \( M^{**} \cdot T \). \( K_1 k_2 [ATP] \) is proportional to the ATP concentration. If, in addition, the formation of \( M^{**} \cdot T \) is responsible for only about 25% of the fluorescence enhancement and \( k_{-2} \) is much less than \( k_3 \), this scheme can explain the data presented in Fig. 3.

Scheme 7 can be tested by comparing the rates of the fluorescence enhancement, irreversible ATP binding, and the initial Pi burst. Of course, this comparison must be made where the rate of the fluorescence enhancement is independent of ATP concentration. Under conditions where the rate of the fluorescence enhancement is limited by ATP concentration, all three rates will be identical as shown in Fig. 4 of the accompanying paper (3). However, at high ATP...
Fig. 4. Comparison of the rate of the irreversible ATP binding, the rate of the initial Pi burst and the rate of the fluorescence enhancement under the conditions shown by the arrow in Fig. 3A. The normalized time course of the respective rates; B, first order plots of the time courses represented in A. The irreversible ATP binding (●) and the initial Pi burst (△) were measured in the three-syringe, quenched flow apparatus, whereas the fluorescence enhancement (○) was measured in the two-syringe, stopped flow apparatus. The concentrations of S-l and ATP were 20 and 80 μM, respectively. The rates observed were greater than 100 s⁻¹ for the irreversible ATP binding, 20 s⁻¹ for the initial Pi burst, and 23 s⁻¹ for the fluorescence enhancement.

concentration, where the rate of the fluorescence enhancement is independent of the ATP concentration, if, as assumed by Bagshaw et al. (15), the rate of the initial Pi burst is much faster than k₂. Scheme 2 predicts that the rate of irreversible ATP binding and the rate of the fluorescence enhancement will be identical. Scheme 7, on the other hand, predicts that the rate of irreversible ATP binding will be faster than the rate of the fluorescence enhancement which should be essentially equal to the rate of the initial Pi burst.

Fig. 4 shows the rate of irreversible ATP binding, the rate of the fluorescence enhancement and the rate of the initial Pi burst at 80 μM ATP where, as shown in Fig. 3, the rate of the fluorescence enhancement is maximal. As can be seen, the rate of irreversible ATP binding is considerably faster than the rate of the fluorescence enhancement or the rate of the initial Pi burst. On the other hand, the rates of the fluorescence enhancement and the initial Pi burst are nearly identical with the rate of the initial Pi burst being only about 20% lower than the rate of the fluorescence enhancement. The irreversible ATP binding process is 82% complete within 13 ms (Fig. 4A) which corresponds to a kₘₐₓ > 100 s⁻¹. This is significantly faster than the kₘₐₓ of 20 and 23 s⁻¹ obtained for the Pi burst and fluorescence enhancement, respectively. This experiment was repeated numerous times under this condition and the rate of irreversible ATP binding was always found to be considerably faster than the rate of the initial Pi burst and the fluorescence enhancement. It should be pointed out that, as shown in Fig. 4B, the rates of both the irreversible ATP binding and the initial Pi burst tend to decrease somewhat during the last 20% of the reaction. This decrease in rate was usually observed, although a similar decrease in the rate of the fluorescence enhancement was not observed. It is possible that the decrease in the rates of the irreversible ATP binding and the initial Pi burst are due to partial denaturation of a small amount of S-1. However, this small deviation does not invalidate the basic finding that the rate of the irreversible ATP binding is considerably faster than the rate of the fluorescence enhancement which, in turn, is nearly equal to the rate of the initial Pi burst as predicted by Scheme 7.

If most of the fluorescence enhancement is due to the formation of M**•• D-P, rather than the formation of M*• T, then the magnitude of the fluorescence enhancement should be similar at low ATP concentration where both the formation of M*• T and M**•• D-P, is detected, and at high ATP concentration where the formation of M*• T becomes so fast that it is not detected due to the dead time of the stopped flow apparatus so that only the formation of M**•• D-P is observed. Fig. 5 shows that, as predicted by Scheme 7, the magnitude of the fluorescence enhancement is only about 25% lower at high [ATP] than at low [ATP], suggesting that about 75% of the fluorescence enhancement is indeed due to the formation of M**•• D-P.

If Scheme 7 is correct, then at an ATP concentration where the rate of the fluorescence enhancement is about half its maximal value, a lag should be observed in both the rates of the fluorescence enhancement and the initial Pi burst, whereas the rate of the irreversible ATP binding should not show a lag. In fact, Bagshaw et al. (15) previously reported that a lag occurred in the initial Pi burst. The lag in the rates of the initial Pi burst and the fluorescence enhancement is expected because at relatively low ATP concentration, the hydrolysis step (M*• T \rightleftharpoons M**•• D-P) with a rate constant, k₁ + k₃, cannot occur until after the relatively slow second order binding of ATP takes place (M + T \rightleftharpoons M*• T) with an observed rate constant of K₄k₅[ATP]. Fig. 6 shows that a lag in the fluorescence enhancement and the initial Pi burst does indeed occur. The lag in the rate of the fluorescence enhancement is slightly smaller than the lag in the initial Pi burst. This is probably due to the small fluorescence increase which occurs on formation of M*• T and the data treatment does not take this into account. Nevertheless, the observation of a lag in the fluorescence enhancement is strong evidence that the
increase in fluorescence is associated mainly with the hydrolysis of ATP rather than the irreversible binding of ATP.

The comparisons of the rate of irreversible ATP binding, the rate of the initial P, burst and the rate of the fluorescence enhancement shown in Figs. 4 and 6 were performed at very low ionic strength. It is of interest to determine whether at higher ionic strength as well, the fluorescence enhancement is associated with the initial P, burst rather than the binding of ATP. However, there is a difficulty in making this determination at higher ionic strength. On the basis of Scheme 7, only

![Diagram](http://www.jbc.org/)

at an ATP concentration where \( K_{b2}[\text{ATP}] \gg k_3 + k_{-3} \) will the rate of the fluorescence change plateau at its maximal value, \( k_3 + k_{-3} \). But, as the KCl concentration is increased to 0.1 M at pH = 7, 15°C, \( K_{b2} \) (the second order binding constant) shows about a 3-fold decrease, while \( k_3 + k_{-3} \) (the rate of the initial P, burst) shows about a 2-fold increase (Table I). Therefore, the ATP concentration must be in the millimolar range before the maximal fluorescence rate is achieved. Since the initial P, burst cannot be accurately measured at ATP concentrations in great excess of the S-1 concentration due to a high blank at this high \( [\gamma-\text{32P}]\text{ATP} \) concentration, a comparison of the maximal rate of the fluorescence increase and the rate of the initial P, burst cannot easily be performed at pH 7, 0.1 M KCl. However, as shown in Fig. 7, at pH 6.4, 0.1 M KCl, \( k_3 + k_{-3} \) is lower than at pH 7 so that the maximal rate of the fluorescence enhancement occurs at a relatively low ATP concentration. Therefore at pH 6.4, we can compare the rate of the initial P, burst, the rate of irreversible ATP binding, and the rate of the fluorescence enhancement.

![Table I](http://www.jbc.org/)

The effect of salt, temperature, and pH on the values of \( K_{b1} \) and \( k_3 + k_{-3} \)

All the experiments were done using S-1. In cases where HMM or myosin were also used, the rates were not found to be significantly different from those obtained using S-1. The value for \( K_{b1} k_3 \) is extracted from the slope of a plot of \( k_{on} \) versus ATP, e.g. Fig. 1A, where the concentration of ATP is low and the \( k_{on} \) is proportional to the ATP concentration added. The value of \( k_3 + k_{-3} \) at 0.5 M KCl, 20 and 15°C, pH 8, could not be accurately determined since the \( k_{on} \) did not reach a maximum value even at 2 mM ATP and at this high concentration of ATP, the magnitude of the fluorescence enhancement becomes very small partly because of the absorption of the excitation light by the ATP and partly because a portion of the magnitude is lost due to the dead time of the stopped flow apparatus.

![Fig. 6](http://www.jbc.org/)

FIG. 6. Time courses of the irreversible ATP binding, the initial P, burst, and the fluorescence enhancement at low ATP concentration. A, normalized time course for each measurement; B, first order plots of the curves in A. ○, irreversible ATP binding; △, initial P, burst; ○, fluorescence enhancement. The reaction medium contains 1 μM S-1, 4 μM \( [\gamma-\text{32P}]\text{ATP} \), 1.5 mM MgCl₂, 7.8 mM imidazole, pH 7, 15°C. The apparent rate constants from B are 20 s⁻¹ for irreversible ATP binding and 11 s⁻¹ for both the initial P, burst and the fluorescence enhancement.

![Fig. 7](http://www.jbc.org/)

FIG. 7. Dependence of the rate of the fluorescence enhancement on the ATP concentration at 0.1 M KCl, 2 mM MgCl₂, 20 mM 1,4-piperazinediethanesulfonic acid, pH 6.4, 15°C. The concentration of S-1 was kept at 2 μM. The apparent second order binding constant was estimated to be 10⁴ M⁻¹ s⁻¹. Arrow, ATP concentration at which the rates for irreversible ATP binding, the initial P, burst, and the fluorescence enhancement are compared in Fig. 8.

![Fig. 8](http://www.jbc.org/)

FIG. 8. Comparison of the irreversible ATP binding, the initial P, burst, and the fluorescence enhancement under the conditions shown by the arrow in Fig. 7. The concentrations of S-1 and ATP used were 20 and 80 μM, respectively. The normalized time courses of A are plotted as first order plots in B. ○, irreversible ATP binding; △, initial P, burst; ○, fluorescence enhancement. The rates observed were greater than 100 s⁻¹ for the irreversible ATP binding, 22 s⁻¹ for the initial P, burst, and 26 s⁻¹ for the fluorescence enhancement.

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* HMM was also used.

* Myosin was also used.
enhancement at relatively high ionic strength. Fig. 8 shows this comparison at the ATP concentration marked by the arrow in Fig. 7. As can be seen, the same result was obtained under this condition as was found at lower ionic strength at pH 7. The irreversible binding of ATP is considerably faster than the rate of the fluorescence enhancement which in turn is nearly identical to the rate of the initial P\textsubscript{i} burst. Therefore, even at relatively high ionic strength, the fluorescence increase appears to accompany the hydrolysis of ATP in the initial P\textsubscript{i} burst rather than the conformational change induced by the binding of ATP.

**DISCUSSION**

In this paper we have investigated the cause of the fluorescence increase which accompanies the binding of ATP to myosin. Our initial experiments ruled out the mechanism involving an isomerization of the myosin prior to ATP binding since such a mechanism required the k\textsubscript{a}, to be directly proportional to the concentration of myosin when the experiments are carried out with the condition that [myosin] \gg [ATP]. In fact, the rate of the fluorescence increase plateaus at high protein concentration just as the rate of the fluorescence increase plateaus at high ATP concentration. Therefore the process which is responsible for the fluorescence enhancement must occur subsequent to the binding of ATP.

The fundamental pre-steady state kinetic studies of Trentham and his collaborators (6, 8, 11, 15, 16) have demonstrated; first, that the binding of ATP is essentially irreversible; second, that a rapid equilibrium occurs between M\textsuperscript{*}.T and M\textsuperscript{**}.D-P\textsubscript{n} which is responsible for the 18O exchange observed with myosin; and third, that the rate-limiting step of the myosin ATPase is a conformational change occurring after the initial P\textsubscript{i} burst but before product release. They also found that both with ATP and several ATP analogues the rate of the fluorescence change levels off at high nucleotide concentrations and on this basis they suggested the following mechanism for the fluorescence increase:

\[
\begin{align*}
\text{M + T} & \overset{k_1}{\underset{k_{-1}}{\rightleftharpoons}} M\cdot T \\
\text{~50\% fluorescence} & \uparrow
\end{align*}
\]

\[
M + T \overset{k_1}{\underset{k_{-1}}{\rightleftharpoons}} M\cdot T \quad \overset{k_2}{\underset{k_{-2}}{\rightarrow}} M\textsuperscript{*}.T
\]

\[
\text{~50\% fluorescence} \uparrow
\]

\[
M + T \overset{k_3}{\underset{k_{-3}}{\rightarrow}} M\textsuperscript{**}.D-P
\]

where k\textsubscript{1} > k\textsubscript{2}, k\textsubscript{-1} \approx 0, and k\textsubscript{-2} \approx k\textsubscript{3}. In this model, although a fluorescence increase accompanies the hydrolysis of ATP, the rate of the fluorescence change depends only on k\textsubscript{1} and k\textsubscript{2}; since k\textsubscript{1} > k\textsubscript{2}, Taylor (5) and Sleep and Taylor (12) modified this model slightly by suggesting that all of the fluorescence increase occurred in Step 2 of the scheme:

\[
\text{100\% fluorescence} \uparrow
\]

\[
M + T \overset{k_1}{\underset{k_{-1}}{\rightleftharpoons}} M\cdot T \overset{k_2}{\underset{k_{-2}}{\rightarrow}} M\textsuperscript{*}.T \overset{k_3}{\underset{k_{-3}}{\rightarrow}} M\textsuperscript{**}.D-P
\]

They made this modification because they found k\textsubscript{1} + k\textsubscript{-1} to be less than k\textsubscript{3} but the rate of the fluorescence enhancement still appeared to follow a single exponential function. Both of these models predict that 1) the rate of the fluorescence enhancement will proceed via a single exponential, 2) the observed rate constant will follow a hyperbolic function with respect to ATP concentration, 3) the rate of irreversible ATP binding will be equal to the rate of the fluorescence enhancement, and 4) the initial P\textsubscript{i} burst will occur with at least a slight lag after the fluorescence increase. In support of these models, Taylor (5) and Sleep and Taylor (12) presented data showing that the rate of irreversible ATP binding was equal to the rate of the fluorescence enhancement, which fitted a simple exponential. In addition, they found that the rate of the initial P\textsubscript{i} burst was about half the rate of the fluorescence enhancement.

The data presented in this paper are in disagreement with the data of Taylor (5) and Sleep and Taylor (12), and suggest that the models for the fluorescence enhancement proposed by Bagshaw and Trentham (11) and Taylor (5) require modification. First, at pH 7 in the absence of KCl (Fig. 3), we find that the observed rate constant for the fluorescence enhancement is not hyperbolically related to the ATP concentration as these models predict. Second, and most important, our results clearly show that the rate of irreversible ATP binding is considerably faster than both the rate of the fluorescence enhancement and the rate of the initial P\textsubscript{i} burst. Finally, we find that the rate of the fluorescence enhancement is similar to the rate of the initial P\textsubscript{i} burst. Frequently, we find that the initial P\textsubscript{i} burst tends to be about 15% slower than the rate of the fluorescence enhancement. However, because these two measurements are carried out on different instruments and are both very temperature-dependent, it is likely that this small difference is within the range of experimental error.

Recently, in agreement with the results presented in this paper, Johnson et al. (13) have also reported that a significant amount of the fluorescence increase is associated with the initial P\textsubscript{i} burst and that the maximal rate of the fluorescence enhancement is equal to the rate of the initial P\textsubscript{i} burst.

On the basis of the data presented in this paper, we conclude that most of the fluorescence enhancement induced by ATP is not due to the conformational change which occurs when ATP binds but rather to the subsequent hydrolysis of the ATP in the initial P\textsubscript{i} burst. Thus the model of Bagshaw et al. (8) (Scheme 1) in effect becomes:

\[
\begin{align*}
\text{~25\% fluorescence} & \uparrow \\
M + T & \overset{K_{k_1}}{\underset{k_{-1}}{\rightarrow}} M\cdot T \\
\text{~75\% fluorescence} & \uparrow
\end{align*}
\]

\[
M + T \overset{k_3}{\underset{k_{-3}}{\rightarrow}} M\textsuperscript{**}.D-P
\]

where k\textsubscript{-1} \approx k\textsubscript{3}, k\textsubscript{3} \approx k\textsubscript{1}, and M\textsuperscript{*}.T shows only a small fluorescence increase compared to the formation of M\textsuperscript{**}.D-P\textsubscript{n}. In this model the rate of irreversible ATP binding is K\textsubscript{k1}k\textsubscript{2} while the maximal rate of both the fluorescence increase and the initial P\textsubscript{i} burst is k\textsubscript{1} + k\textsubscript{-1}. As in the model of Bagshaw et al. (8), in this model two steps are required for the development of fluorescence change, the second order binding of ATP (K\textsubscript{k1}) followed by a conformational change due to the hydrolysis of ATP, (k\textsubscript{1} + k\textsubscript{-1}). But, in this model, the second order binding step is irreversible. Therefore, this model is consistent with the data which show that at low ATP concentration the rate of the fluorescence increase is not a simple exponential process (Fig. 6) and that the observed rate constant for the fluorescence enhancement does not show a simple hyperbolic dependence on ATP concentration (Fig. 3B).

In presenting this model, we have assumed that the second order binding of ATP to myosin is, itself, a two-step process involving, first, the equilibrium formation of the collision intermediate M\cdot T and then an irreversible conformational change to form M\textsuperscript{*}.T. Thus, the second order rate constant for ATP binding is K\textsubscript{k1}k\textsubscript{2} rather than just k\textsubscript{1}. Of course, from our own data we have no direct evidence for a two-step
Fluorescence Enhancement and the Initial P1, Burst of Myosin

binding process for ATP. Because the irreversible binding of ATP must be measured in the quenched flow apparatus, the shortest reaction time which could be obtained was about 12 ms. At this time more than 80% of the myosin forms a complex with ATP, suggesting that, at 80 μM ATP, the pseudo first order rate constant for M* . T formation (Kk2[ATP]) is about 100 s⁻¹, i.e. Kk2 ~ 1 × 10⁶ M⁻¹ s⁻¹. This is somewhat slower than expected on the basis of the second order rate constant for ATP binding (Kk2) obtained from the rate of the fluorescence enhancement measured at very low ATP concentration. This measurement gave a value for Kk2 of 3 × 10⁶ M⁻¹ s⁻¹. Thus, it is possible that the pseudo-first order rate constant for M* . T formation is beginning to level off at high ATP concentration. However, within the limits of accuracy of the measurement we cannot be certain of this. Therefore, we cannot directly determine whether the binding of ATP is a two-step process nor can we determine the value of k2. However, a two-step binding process is certainly not unlikely since a value for Kk2 of about 1 × 10⁶ is at least 1 order of magnitude lower than the value expected for a diffusion controlled reaction (16, 17). Furthermore, Bagshaw et al. (8) have shown that both ATPyS and ADP bind to myosin in two steps and therefore it seems very likely that ATP also binds in this way.

In this regard, it is useful to consider how our results with ATP relate to the studies of Bagshaw et al. (8) using ADP and ATPyS. At 20°C, 0.1 M KCl, pH = 7, they measured, as a function of nucleotide concentration, the rates of the fluorescence change induced by ATPyS, which is not hydrolyzed in an initial P1, burst, and ADP, which, of course, is not hydrolyzed at all. They found that, for both nucleotides, the rates of the fluorescence change showed a dependence on nucleotide concentration and had maximal values very similar to the equivalent parameters observed with ATP. On the basis of these fluorescence data, Bagshaw et al. suggested that k2 was similar for ATPyS, ADP, and ATP. However, our results strongly suggest that the irreversible binding of ATP occurs faster than the fluorescence change, suggesting that k2 cannot be determined by measuring the maximal rate of the fluorescence change. Thus, it could be that k2 is much faster for ATP than for ADP and ATPyS but, under the conditions used by Bagshaw et al., the maximal rate of the fluorescence change is the same for the three nucleotides because coincidentally, under these conditions, k2 for ADP and ATPyS is approximately equal to k2 + k3 for ATP. A lower value of k2 for ADP compared to ATP would not be surprising considering the marked difference between the binding constants of ADP and ATP to myosin, about 10⁶ (18) and 10⁻¹ (19, 20), respectively. The binding constant of ATPyS to myosin is not known, but it is certainly not unlikely that replacement of an oxygen with a sulfur on the terminal phosphate of the ATPyS could decrease the value of k2. Furthermore, the larger magnitude of the fluorescence enhancement observed with ATP compared to ADP and ATPyS is consistent with the view that the fluorescence enhancement induced by ATP involves hydrolysis of the ATP (formation of M* . D-P), while the fluorescence change induced by ADP and ATPyS is related only to the binding of these nucleotides.

To test this view directly, it would be necessary to compare the rates of irreversible ATP binding and the maximum rate of the fluorescence change at 20°C, 0.1 M KCl, pH = 7, the conditions used by Bagshaw et al. (8). Unfortunately, as discussed under “Results,” this is not possible to accomplish experimentally at the present time. However, we were able to perform this experiment under the not too dissimilar conditions of 15°C, 0.1 M KCl, pH = 6.4 (Fig. 8) and here our results strongly suggest that the rate of irreversible ATP binding is, indeed, considerably faster than the maximal rate of the fluorescence enhancement. It, therefore, appears that, for ATP, the maximal rate of the fluorescence enhancement represents the rate of the initial P1, burst (k3 + k3), while for ADP and ATPyS it represents the rate of the conformational change, k2.

Another observation which we consistently made in regard to both the irreversible ATP binding and the initial P1, burst was that the last 20 to 30% of the reaction appeared to occur at a somewhat lower rate. If this is due to a small fraction of S-1 which still binds ATP irreversibly but at a somewhat lower rate, it might partially account for the unexpectedly high values for the Kd of the rabbit myosin ATPase observed by Taylor (0.25 μM) (5) and Taylor and Weeds (2.6 μM) (7). On the basis of Scheme 1, Kd = k5k6/(k5 + k -3)Kk2 (8). Therefore, if Kk2 were low for some of the S-1, it might tend to increase the observed value of Kd. Further work will be required to determine if this is indeed the explanation for the observation that the Kd of the myosin ATPase is about 1 order of magnitude higher than expected on the basis of Scheme 1.

Our finding that the fluorescence increase is mainly associated with the initial P1, burst rather than the binding of ATP is of interest in regard to the mechanism of the actomyosin ATPase. Both Chock et al. (14) and Sleep and Taylor (12) observed that a fluorescence increase occurred following the dissociation of acto-S-1 by ATP. Since Taylor had suggested that no fluorescence change was associated with the initial P1, burst, Sleep and Taylor (12) postulated that a new state, M1 . T, occurred immediately after dissociation of the acto-S-1 by ATP and then the fluorescence increase was due to the transition from M1 . T to M* . T.

\[
AM + T \rightleftharpoons AM . T \rightleftharpoons M1 . T + A \rightleftharpoons M* . T + A \tag{11}
\]

On the other hand, Chock et al. (14) suggested that the fluorescence increase which occurs following dissociation of the acto-S-1 by ATP was due to the initial P1, burst:

\[
AM + T \rightleftharpoons AM . T \rightleftharpoons M* . T + A \rightleftharpoons M* . D-P + A \tag{12}
\]

The demonstration in this paper that a marked increase in fluorescence is associated with the initial P1, burst strongly supports the mechanism of Chock et al. (14) and makes it unnecessary to postulate the state M1 . T.

The data presented in this paper clarify one other feature of the actomyosin ATPase mechanism. The results of Chock et al. (14) and Sleep and Taylor (12) showed that the rate of dissociation of AM by ATP, i.e. the rate of formation of M* . T in Scheme 10, was very rapid. In fact, this rate appeared to be much faster than the rate of M* . T formation from M + T since the maximal rate of the fluorescence increase was relatively slow with S-1 alone and this rate was thought to be the maximal rate of M* . T formation in the absence of actin. However, the data presented in this paper suggest that k3, the maximal rate of formation of M* . T from M + T, may in fact be as fast as the rate of formation of M* . T from AM + T. Thus, it may not be necessary to postulate that actin affects the maximal rate of formation of M* . T.

Table I summarizes under varied conditions our values for the second order binding constant of ATP to myosin (Kk2) and the rate of the initial P1, burst, i.e. the maximal rate of the fluorescence change (k3 + k3). As can be seen, Kk2 is relatively insensitive to changes in temperature or pH but does tend to decrease markedly as theionic strength is increased. On the other hand, k3 + k3 increases markedly with increasing temperature or pH and, to a lesser extent, with increasing ionic strength. One of the key questions about the mechanism of the actomyosin ATPase is the nature of the rate-limiting
step, that is whether the initial Pi burst is identical to the rate-limiting transition from the refractory to the nonrefractory state (14). Obtaining the rate of the initial Pi burst from the maximal rate of the fluorescence increase should be very helpful in solving this problem. In this regard, preliminary data (21) suggest that at 15°C, the rate of the initial Pi burst is considerably faster than the maximum actin-activated ATPase rate. Therefore, at least at 15°C, the initial Pi burst does not appear to be the rate-limiting step in the actomyosin ATPase cycle.

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