Intermediate States of Actomyosin Adenosine Triphosphatase†

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ABSTRACT: The early kinetic steps of actomyosin subfragment 1 (acto-S1) adenosine triphosphatase have been investigated by simultaneous monitoring of fluorescence and light scattering and also by observation of the time course of the production of phosphate. The results show that fluorescence enhancement occurs after the dissociation of actomyosin and that the rate of enhancement is similar to the maximum rate of enhancement for S1 alone, under similar conditions of pH and temperature. The maximum rate of the phosphate burst for acto-S1 is also approximately the same as that for S1 alone. The maximum rates for fluorescence enhancement or phosphate formation are reached at much lower adenosine triphosphate concentrations for acto-S1 than for S1. An extension of the actomyosin scheme is presented which accounts for these results.

The work of Lymn and Taylor (1971) on the dissociation of acto-HMM[1] by ATP, and the rate of ATP splitting by acto-HMM, showed that at 20 °C, pH 8.0, dissociation was considerably faster than ATP hydrolysis. Thus, it was concluded that dissociation must take place prior to ATP hydrolysis, and that the hydrolysis step occurs while myosin is dissociated from actin. Based on these findings and a previous study of the myosin phosphate burst (Lymn and Taylor, 1970), a scheme was proposed for actomyosin ATPase which could be correlated with the steps in the contractile cycle as described by Huxley and others (Huxley, 1969). Further studies of myosin ATPase (Bagshaw et al., 1974; Koretz and Taylor, 1975) have expanded the simple scheme to include a conformation change which precedes the hydrolytic step and affects the rate at which this step occurs.

SCHEME I.

$$M + ATP \overset{(1)}{\rightleftharpoons} MATP \overset{(2)}{\rightleftharpoons} MATP^{**} \overset{(3)}{\rightleftharpoons} MPr^{**} \underset{\text{step}}{\overset{\text{rate limiting}}{\rightleftharpoons}} M + \text{products}$$

It is necessary to determine the relationship of the actomyosin scheme to this more complex myosin mechanism. The kinetic studies presented here show that fluorescence enhancement occurs after dissociation, and the hydrolysis step at pH 6.9 and 3 °C appears to be slower than the fluorescence step. The results confirm the postulate of the Lymn–Taylor mechanism that dissociation of actomyosin precedes the hydrolysis of ATP and the evidence is consistent with a simple extension of this mechanism.

Materials and Methods

Heavy meromyosin and S1 were prepared by the method of Weeds and Taylor (1975). Actin was prepared by the method of Hitchcock (1973) and was essentially free of tropomyosin, as determined by sodium dodecyl sulfate gel electrophoresis.

Light scattering and fluorescence were measured simultaneously by two photomultipliers at right angles to the incident beam using a stopped-flow apparatus constructed in this laboratory. The transmitted beam was monitored by a photodiode (EG&G HUV 4000B). Digital data from both photomultipliers were stored and analyzed using a PDP8/I computer.

The intensity of light scattered by acto-S1 at 295 nm is considerably larger than the fluorescence emission at 340 nm, and the intensity changes by 50% during dissociation while the change in fluorescence is 10–20%. There is a small overlap in the pass bands of the filters used to excite and isolate the fluorescence. The changes in scattering and fluorescence oppose each other and the apparent fluorescence signal can decrease during the dissociation phase if scattered light is not efficiently filtered. At high ATP concentration, as will be described, dissociation is essentially complete before there is an appreciable change in fluorescence; consequently, scattering effects do not hamper measurement of the rate constant of the fluorescence process. However, useful information is obtained by measuring the concentration dependence. In the first series of experiments, light scattering was significantly reduced by polarizing the incident beam in the perpendicular plane with a Glan-Thompson prism or observing the fluorescence beam after passage through a horizontal polarizer (Polacoat UV polarizing sheet). Some light scattering remained and the signal was fitted to two exponential terms by a computer program in order to obtain the rate constant associated with the fluorescence change.

In later work, the observation cell was redesigned to reduce stray light and the fluorescence signal was isolated by a sharp cutoff UV filter (Ealing Optics 26-4655) and a 340-nm interference filter (Optics Technology). The light scattering signal was isolated with a 290-nm interference filter. The change in voltage on the same photomultiplier with identical gain settings was compared for the association of actin with S1 using the cutoff and 290-nm filter and the reaction of the same S1 solution with ATP using the cutoff and 340-nm filter. In the first case, the change in voltage arises from light scattering, which passes the cutoff filter, while the second signal is light scattering plus fluorescence. The ratio of voltage changes was less than 0.03, indicating that direct light-scattering effects are negligible.

A further error arises in comparing the magnitudes of flu-

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[1] Abbreviations used are: acto, actomyosin; HMM, heavy meromyosin, S1, subfragment 1; ATP, adenosine triphosphate; ATPase, adenosine triphosphatase.

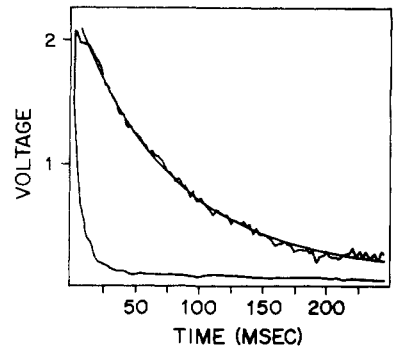


FIGURE 1: Light scattering and fluorescence transients for the reaction of acto-S1 with ATP. Upper trace, fluorescence signal (decreasing voltage corresponds to increasing fluorescence); the irregular line is the experimental signal, the smooth curve is the computer generated fit to a single exponential (12.7 s^{-1}) commencing at 10 ms. Lower trace is the light-scattering signal. Experimental conditions: pH 7.3, 3 °C, 40 mM KCl, 0.05 mM ATP, 5 μM S1, 7.5 μM actin.

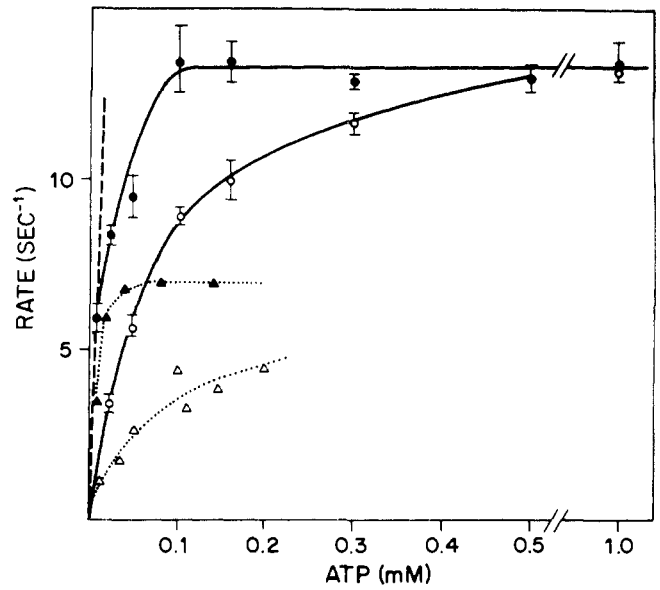


FIGURE 2: Rates of dissociation, fluorescence enhancement, and ATP hydrolysis of acto-S1 and S1 as a function of ATP concentration. Experimental conditions: pH 6.9, 3 °C, 50 mM KCl, 5 mM $MgCl_2$. Dashed line: rate of dissociation determined by light scattering. Rate of fluorescence enhancement of acto-S1, (●) and solid line; rate of fluorescence enhancement of S1, (○) and solid line. Error bars refer to standard deviation of the mean of five traces. Rate of hydrolysis (early burst) for acto-S1 or acto-HMM, (▲) and dotted line; rate of hydrolysis for S1 or HMM, (△) and dotted line.

orescence enhancement of acto-S1 with S1 on binding ATP. In most experiments, the actin to S1 ratio was 1.5:1 to insure that all the S1 is initially bound to actin. Actin contributes about one-third of the static fluorescence signal and the relative voltage signal, $\Delta V/V_f$, is, of course, smaller. The relevant quantity is the absolute voltage change, ΔV, which is proportional to incident light intensity. Even at high ATP concentrations such that dissociation is complete before the change in fluorescence, the turbidity of actin plus S1 is slightly higher than S1 alone. Measurements were made in a 2-mm path length cell and transmission was 0.85–0.95. The transmitted beam was measured to allow a correction for differences in incident light intensity, which is 2–3% under the conditions used.

Phosphate Analysis. The hydrolysis of [γ-^{32}P]ATP was measured by a quench-flow apparatus, which is described elsewhere (Taylor, 1976). It was used in a direct drive mode or the reaction solution was held in a delay line and mixed with acid after a time interval determined by an electronic timer and relay. A time range from 10 ms to 10 s could be covered conveniently.

Radioactive phosphate was measured as described previously (Lymn and Taylor, 1970). The ATP–protein ratio was selected to give 10–25% hydrolysis during the transient phase to reduce the error from the size of the zero-time blank (2%).

Results and Discussion

Rate of Fluorescence Enhancement of Acto-S1 and S1. The time course of fluorescence enhancement and light scattering for the reaction of acto-S1 at a moderate ATP concentration is shown in Figure 1 (0.05 mM ATP, 40 mM KCl, pH 7.3, 3 °C, 5 μM S1, 7.5 μM actin final concentrations). The smooth curve drawn through the fluorescence data is the best fit to a single exponential obtained by the analysis program (k = 12.7 s^{-1}). It is evident that dissociation, as measured by light scattering, is considerably faster than the fluorescence step. The rate constant, obtained using a faster sweep rate, is 118 ± 15 s^{-1}. The fluorescence trace shows a departure from an exponential for the first 10 ms of the sweep, possibly caused by the change in turbidity of the solution. The effect is small, about 5% of the total signal, and the rate constant was determined by starting the fitting program at the end of the perturbation.

The rate of dissociation at 0.1 mM ATP was 350–400 s^{-1}; thus, the scattering signal is complete in less than 10 ms. For ATP concentrations of 0.1–1.5 mM, the fluorescence signals are essentially separated from any perturbation from light scattering. Over this range, acto-S1 gave rate constants of 19 ± 1 s^{-1}, while the maximum rate for S1 was 18.6 ± 0.1 s^{-1} (1 mM ATP, pH 7.3). The rate constant was measured for actin to S1 mole ratios of 1:1, 1.5:1, and 2:1 to establish that the parameter was a property of acto-S1. Similar results were obtained, except for the decrease in the fractional change of fluorescence with increasing actin ratio. Turbidimetric titration of S1 with actin and measurements of the association constant (Highsmith, 1976) indicate essentially complete binding of S1 at a concentration of 5 μM with actin in small excess. A ratio of 1.5:1 and an S1 concentration of 5 μM after mixing was selected for kinetic studies.

The maximum rate of the acto-S1 fluorescence change increased with pH between 6.5 and 8.5 and gave the same pH dependence as S1 within an uncertainty of ±10%. The rate also increased with temperature in parallel with the increase for S1 alone. At 20 °C, pH 7, the rates were 153 ± 8 and 150 ± 6 s^{-1} for acto-S1 and S1, respectively. The rate of dissociation measured by light scattering exceeded 500 s^{-1} without reaching a plateau either at 20 or 3 °C. From the equality of the maximum rates of the fluorescence change of acto-S1 and S1 for a range of conditions and the much faster rate of dissociation, it is concluded that dissociation occurs before the fluorescence step.

Concentration Dependence of the Rate of Fluorescence Enhancement of Acto-S1 and S1. The concentration dependence was investigated at 3 °C and pH 6.9 (Figure 2). The rates of the fluorescence change are equal at high ATP concentrations, but for acto-S1 the rate does not decrease until the ATP concentration is below 0.1 mM. Further reduction of the ATP concentration gave a sharp decrease in rate. In contrast, the rate for S1 follows a hyperbolic concentration dependence

with an apparent association constant of approximately 10^4 M^{-1}. For low ATP concentrations (10 μM), the rates for dissociation and fluorescence are comparable and the time course of fluorescence may depart from a single exponential. As there are scattering and turbidity effects superimposed, the data were fitted to one exponential process.

Amplitude of the Fluorescence Change for Acto-S1 and S1. A further question is whether the complete fluorescence increase of 20% characteristic of ATP binding to S1 occurs after dissociation or whether some fluorescence change occurs prior to or with the dissociation step. Measurements of fluorescence in the presence of light scattering require both that the scattering from the incident beam be filtered out of the fluorescence channel and that a correction be made for the change of intensity of the exciting light and the change in scattering of the fluorescent beam within the cell.

The fluorescence signal in Figure 1 exhibits a small jump of about 5% during dissociation, indicating that light scattering makes only a small contribution to the apparent fluorescence signal. This effect is absent at ATP concentrations greater than 0.1 mM. To assess the turbidity effects, the intensity of the transmitted beam was measured. Typical values of the transmission relative to buffer are 0.86, 0.93, and 0.96 for acto-S1, actin plus dissociated S1, and S1 alone (7.5 μM actin, 5 μM S1, 2-mm path length). The quantity to be measured is the change in voltage for acto-S1 vs. S1 at a constant intensity of the exciting light. The fractional change, $\Delta V/V_f$, where V_f is the voltage at the end of the transient, is smaller for acto-S1, since actin contributes about one-third of the static fluorescence. At high ATP concentrations, the fluorescence signal occurs after dissociation and the transmission is about 3% larger for S1. The difference is small enough that the correction for turbidity needs to be known only approximately and it is assumed that the effective light intensity varies linearly across the cell. Thus, ΔV can be expressed relative to the voltage measured for the transmitted beam and for the transmission figures given above; the acto-S1 signal would be reduced by 1.5% relative to S1. A small correction could also be made for the scattering of the fluorescent beam. The average path length within the cell is less than 2 mm and the wavelength is larger; thus, the loss from scattering by actin plus dissociated S1 should not be more than 1% larger than for S1 alone.

The voltage change was obtained directly from digital data stored by the computer and the transmitted light intensity was read from a digital voltmeter at the end of the transient. The solutions of S1 and acto-S1 containing the same concentration of S1 were measured alternately for a range of ATP concentrations from 0.1 to 1.5 mM. There was no significant variation with concentration. The ratio of the voltage change of acto-S1 to S1 was 1.02 ± 0.05 uncorrected for turbidity effects and 1.04 ± 0.08 applying the corrections described above (pH 7.3, 40 mM KCl, 10 mM $MgCl_2$, 7.5 mM actin, 5 μM S1, observation cell concentrations). A similar result was obtained in 0.1 M KCl, pH 8. Thus, the fluorescence change after dissociation is equal in magnitude to the value obtained with S1.

It is difficult to answer the question of whether there is a change in fluorescence on binding of actin to S1. An increase in fluorescence was observed with essentially the same rate constant as the increase in light scattering and a magnitude of about one-fourth that produced by binding ATP. Any scattering of the incident beam which passes the filters will add to the apparent fluorescence signal. The change in turbidity of the solution arises from light scattering, rather than absorption, and a simple correction for the effective intensity of the exciting light cannot be made. Both effects have the same rate constant and concentration dependence as the fluorescence signal. In view of the uncertainties, it can only be concluded that actin binding produces a much smaller fluorescence increase than ATP binding.

Phosphate Burst Experiments. The rapid phase of ATP hydrolysis was measured for acto-HMM and acto-S1 and compared with parallel experiments on HMM or S1 alone. At 3 °C and pH 6.9, the size of the early burst was approximately 0.5/site and somewhat larger, 0.6–0.65/site for acto-HMM or acto-S1. The difference appeared to be larger for HMM and may indicate that, under our conditions at high actin concentrations, the system is not completely dissociated. The problem requires further investigation, rather than a speculative explanation. The lower value of the burst for S1 compared to 0.7–0.8 obtained at pH 8 and 20 °C can be explained by a decrease in the equilibrium constant of the MATP** to MPr** step. A more detailed study of the burst at low temperature is reported elsewhere (Taylor, 1976). The time course at a given ATP concentration gave a moderately good fit to a single exponential, although a lag was detected in some experiments. The rate constants determined are much less accurate than for the fluorescence step and values are probably underestimated, since two exponentials should be used to fit the data.

Higher protein concentrations were used compared to fluorescence measurements in order to maintain a substrate to site ratio of 4 or 5 to 1. At high ATP concentrations ($1–2 \times 10^{-4}$ M), the ATP to site ratio was larger and the measurements were less accurate because the zero-time blank is 25–50% as large as the signal. Thus, the maximum rate cannot be measured accurately for S1 and HMM, but for the corresponding actomyosins a plateau is reached at a much lower ATP concentration. The results are included in Figure 2. The maximum rate for acto-S1 and acto-HMM was 6–7 s^{-1}. At low ATP concentrations, the rate increases in parallel with the rate of dissociation. For S1 and HMM, the rate increases much more slowly with concentration and the plateau was not reached. From a series of burst and chase experiments, the rate was estimated to be 6–7 s^{-1} (Taylor, 1976). Within the relatively large limits set by experimental errors of 15–20%, the maximum rates for myosin and actomyosin are the same and are more than 100 times less than the rate of dissociation. Since the fluorescence and hydrolysis steps are slow compared to dissociation, the first dissociated state in the pathway is a myosin–ATP state but we have not detected a change in fluorescence associated with its formation. In the myosin pathway, the first state, M·ATP, does not have an enhanced fluorescence and the ATP is in rapid equilibrium with ATP in the medium (Bagshaw and Trentham, 1974; Koretz and Taylor, 1975). Experiments were undertaken to determine whether the ATP bound to the state produced by dissociation is exchangeable. At a fairly high ATP concentration, circa 0.1 mM, dissociation is complete at 30 ms while the P_i burst has reached 10–15% of its final value. If the bound ATP is exchangeable, mixing with excess unlabeled ATP at short times should limit the further formation of $^{32}P_i$ to the small amount of MPr** subsequently formed from any M·ATP** present during the transient. In Figure 3, the upper curve (open circles) shows the time course of P_i formation by acto-HMM mixed with 0.09 mM [^{32}P]ATP at an ATP to site ratio of 3:1. At 30 and 60 ms, the solution was mixed with excess ATP and the reaction was stopped by adding acid immediately (within 2 s). The extent of hydrolysis was nearly equal to the complete phosphate burst of 0.6 mol/mol (lower pair of squares). Allowing the reaction to continue for 50 s after mixing with unlabeled ATP raised the total hydrolysis to 0.7 mol/mol (upper pair of squares). The

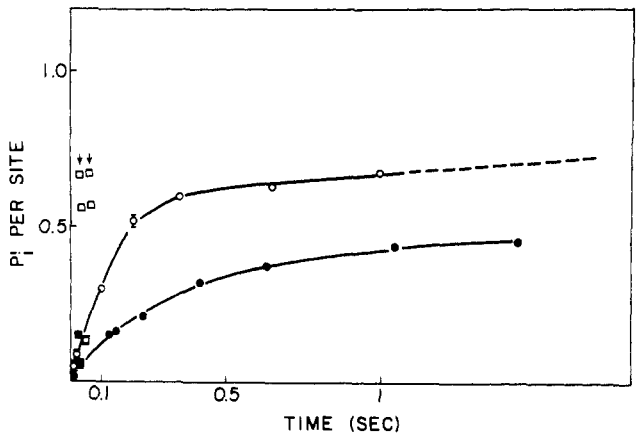


FIGURE 3: Phosphate burst for HMM and acto-HMM. Time course of P_i formation for HMM (solid circles) and acto-HMM (open circles). The acto-HMM reaction was quenched at 30 and 60 ms (arrows) by mixing with a 100-fold excess of unlabeled ATP and was stopped immediately (2-3 s) or after 70 s (open squares). The HMM reaction was quenched at 30 ms and stopped immediately or after 100 s (solid squares). Bars give range of duplicate measurements. Conditions: pH 6.9, 3 °C, 50 mM KCl, 5 mM $MgCl_2$, 3×10^{-5} M HMM sites, 3.2×10^{-5} M actin, ATP/site ratio of 3. The dashed curve is the extrapolation of the steady-state rate.

control experiment with HMM at 30 ms gave an increase of 0.1 mol (solid squares) corresponding to the amount of M·ATP** present. Mixing HMM with excess ATP at the end of the burst phase (1.5 s) and allowing the reaction to reach completion gave a total hydrolysis of 0.8 mol/site. The difference from unity is probably due to the presence of impurities or inactive enzyme plus a small amount of ATP dissociation at low temperature. Since about 90% of the ATP is nonexchangeable, it is concluded that the first dissociated state in the actomyosin pathway is distinct from M·ATP. Similar results were obtained with S-1.

The evidence also suggests that both heads of the HMM of acto-HMM have bound ATP irreversibly in 30 ms. If only 1 ATP was needed to dissociateaacto-MM the second ATP might be expected to bind at the rate characteristic of HMM, in which case less than one-fourth of these sites would be occupied in 30 ms. The total hydrolysis after mixing with unlabeled ATP should be less than 0.5 mol at 50 s. While the problem requires more detailed investigation, the evidence favors 2 ATP, rather than 1 ATP, for dissociation of acto-HMM.

Conclusions

The dissociation of acto-HMM or acto-S1 leads to enhancement of fluorescence at a rate which is similar to that found for HMM or S1 alone, while the rate of dissociation is many times faster. These two rates are clearly separated at pH 6.9 and 3 °C where the rate of dissociation exceeds 700 s^{-1}, while the maximum rate of fluorescence change is 14 s^{-1}. The rate of the ATP hydrolysis step under the same conditions is 6-7 s^{-1}. The magnitude of the fluorescence change following dissociation appeared to be essentially the same as for S1 alone. The results indicate that the conformation change of myosin monitored by fluorescence enhancement occurs after dissociation and the simplest mechanism (ignoring the question of two heads) is:

$$\mathrm{AM + ATP} \overset{(a)}{\rightleftharpoons} \mathrm{AM\,ATP} \overset{(b)-A}{\rightleftharpoons} \mathrm{M\,ATP^{\dagger}} \overset{(c)}{\rightleftharpoons} \mathrm{MATP^{**}} \overset{k_3}{\rightleftharpoons} \mathrm{MPr^{**}}$$

At pH 6.9, 3 °C, 50 mM KCl, $k_{+b} \sim 10^3$ s^{-1}, $k_{+c} = 12$-15 s^{-1}, $(k_{+3} + k_{-3}) = 5$-7 s^{-1}. The state MATP† is defined as a tight ATP binding state but there is no signal associated with it.

The corresponding steps for myosin-ATPase under the same conditions are:

$$\mathrm{M + ATP} \overset{(1)}{\rightleftharpoons} \mathrm{M{\cdot}ATP} \overset{(2)}{\rightleftharpoons} \mathrm{MATP^{**}} \overset{(3)}{\rightleftharpoons} \mathrm{MPr^{**}}$$

with $K_1 = 16 \times 10^3$ M^{-1}, $k_{+2} = 12$-15 s^{-1}, $(k_{+3} + k_{-3}) \sim 5$-7 s^{-1}.

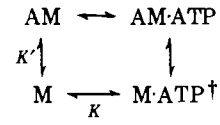
If step a were a single process, the apparent second-order rate constant for ATP binding to actomyosin (10^6 M^{-1} s^{-1}) obtained from the rate of dissociation could be a measure of the actual rate constant. The value is rather low for simple ligand binding and, by analogy with myosin ATPase, the initial binding step is probably a rapid equilibrium (K_a) followed by a conformation change. In this case, the apparent rate constant is K_a times the rate of the conformation change. The maximum rate of dissociation has not been measured but is larger than 500 s^{-1}, which is a lower limit for the rate of the conformation change and for k_b. The value of K_a is approximately 10^3 M^{-1}. The apparent rate constant for acto-S1 (or acto-HMM) is much larger than the value for S1 (or HMM) at pH 6.9 and 3 °C. In the latter case, the quantity measured is $K_1 k_2$ and the two numbers can only be equal by coincidence. However, k_2 has a large dependence on temperature and pH while the rate of dissociation does not (White and Taylor, 1976), and the two rates are similar at pH 8 and 20 °C (Lymn and Taylor, 1971). It should be noted that K_a refers to the initial step in ATP binding and is analogous to K_1 in the myosin scheme, not to the overall ATP binding constant $K_1 K_2$. Analysis of the kinetic behavior does not require specification of the details of steps a and b, since the rate of dissociation is linearly related to substrate concentration.

The concentration dependence of the rate of fluorescence enhancement provides further support for a sequential mechanism. Step b is essentially irreversible at low actomyosin concentrations (<10 μM) and the solution of the rate equations for the steps leading to M·ATP** is

$$[\mathrm{M{\cdot}ATP^{**}}] = [k_c(1 - \exp(-k't)) - k'(1 - \exp(-k_c t))]/(k_c - k')$$

where k' is the rate of dissociation and is proportional to substrate concentration. For ATP concentrations of 0.1 mM or larger, $k' > 100$-200 s^{-1}. Thus, $k' \gg k_c$ and the expression reduces to $(1 - \exp(-k_c t))$. At low ATP concentrations (0.02 mM), k' and k_c are comparable and the fluorescence signal should show a lag. Fitting in this region is not very accurate because turbidity changes introduce some distortion and the data were fitted by a single exponential. It is easily shown by simulation that the procedure will give an average rate constant, which is less than both k' and k_c. In the intermediate concentration range, the apparent rate will increase sharply with ATP concentration. At very low ATP concentrations, such that $k' \ll k_c$, the expression becomes $(1 - \exp(-k't))$, and fluorescence and phosphate rate constants are approximately equal to the rate of dissociation (Figure 2).

The first dissociated state is denoted by the symbol M·ATP†, since it is distinguishable from the nonfluorescent myosin state M·ATP on the basis of ATP exchange and on thermodynamic grounds, since the binding of ATP in this state must be much larger than that of the M·ATP state. The transition from M·ATP† to M·ATP** occurs after dissociation; consequently, the degree of dissociation is determined initially by the association constants of the box.

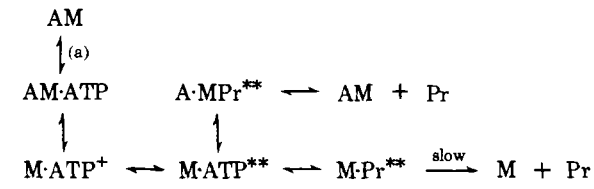


Transient measurements have shown that at least 90% dissociation of 10 μM acto-S1 is produced by 0.1 mM ATP and solution of the multiple equilibria requires $K \geq K'$ to account for this extent of dissociation. The inclusion of a conformation change before dissociation does not affect the conclusion. K' is at least 10^6 M^{-1} (Highsmith, 1976, and unpublished experiments from this laboratory), which gives a lower limit for the binding of ATP in the M·ATP† state, while the value for M·ATP is approximately 10^4 M^{-1}.

Is the state M·ATP† identical with a state of the myosin kinetic scheme? The only other state to consider is M·ATP**. Bagshaw and Trentham (Bagshaw et al., 1974; Bagshaw and Trentham, 1974) have suggested that the fluorescence enhancement of this state is only 10% by analogy with the enhancement of nonhydrolyzed nucleoside triphosphates, AMPPNP and [γ-S]ATP. They refer to the state as M·ATP*. It could be argued that M·ATP* is the first dissociated state and the observed fluorescence change after dissociation is the transition M·ATP* $\rightleftharpoons$ M·Pr**. There would then be a 10% fluorescence increase after dissociation at the rate of the hydrolysis step. The explanation is attractive, since it does not require the introduction of a new myosin state. The following evidence is against this possibility. The fluorescence change after dissociation is 20%, rather than 10%. Phosphate measurements gave a rate for the hydrolysis step, which is half as large as the fluorescence rate. However, the errors in phosphate measurements are large and the rate constant doubles for a 4 °C change in temperature. A 1 or 2 °C temperature error is possible, and the difference in rates might be less than a factor of two but it is unlikely that the errors are so large that the two rates are equal. For this mechanism, the rate of fluorescence enhancement for acto-S1 is $k_3 + k_{-3}$ by hypothesis, while the corresponding experiment for S1 gives k_2. The rates remain equal for a range of pH and temperatures which would require a considerable coincidence. There is no positive evidence that the M·ATP** state has a 10% fluorescence enhancement. Such evidence as there is suggests that the enhancement is essentially the same as M·Pr**, again based on the slower rate of the hydrolysis step at low temperature. Assigning 10 and 20% enhancement to the states would require two exponentials to fit the fluorescence transient and the effect should have been easily observed (this paper). Finally, in experiments in which dissociation overlaps the fluorescence signal, one might expect to detect half the signal at the rate of the dissociation step.

There is some evidence in favor of M·ATP* as the first dissociated state. Chock et al. (1976) have reported similar kinetic evidence at high ATP concentrations, but find approximately a 10% fluorescence change after dissociation. Secondly, there may be some fluorescence enhancement in forming the acto-S1 complex, which, in the simplest interpretation, would require the fluorescence change to be smaller after dissociation. Finally, if M·ATP† is a different state from M·ATP*, the equality of k_c and k_2 is still a coincidence. It should be noted that the conformation change (k_2) has the same rate for ATP, ADP, [γ-S]ATP (Bagshaw et al., 1974), and pyrophosphate (Sleep and Taylor, unpublished proton measurements). There may be a basis for the coincidence in a property of the myosin structure which controls the rate of the transition to the ** state.

The experiments are in quantitative agreement with a sequential scheme. Evidence on the recombination step will be presented elsewhere but for completeness the mechanism is given here:



Step a probably occurs in two stages and the conformation change to the † state is not shown explicitly. The first dissociated state is written as M·ATP†. There are possible objections to the choice of either M·ATP† or M·ATP* as the first dissociated state, and the contribution of actin binding to changes in fluorescence requires further study but the available evidence favors M·ATP†. This state could not be inserted in the main myosin pathway (e.g., M $\rightleftharpoons$ M·ATP $\rightleftharpoons$ M·ATP† $\rightleftharpoons$ M·ATP**) without upsetting the firm experimental evidence that the fluorescence transient fits a single exponential with no lag and shows a hyperbolic dependence of rate on substrate concentration.

The cross-bridge cycle in muscle requires the "orientations" (and probably the conformational states) of the cross bridge to be different for detachment and attachment. The kinetic scheme in solution may provide a basis for this asymmetry, since in the main pathway the dissociation and recombination reactions are separated by at least one and possibly two steps in which a conformation change occurs.

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