Actin Mediated Release of ATP from a Myosin–ATP Complex†

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ABSTRACT: The apparent second-order rate constant, k^a_{-2}, of actin binding to a myosin-ATP state (M*·ATP) and releasing ATP to the medium has been determined by two methods. The first was the measurement of the amount of ATP released when actin was added to the intermediate state, M*·ATP; the second was the measurement of oxygen exchange between medium ATP and HOH. A quantitative treatment of ATP ⇌ HOH exchange is given to allow extraction of elementary rate constants from the data. Agreement between the two methods was good and at low ionic strength and 23 °C, k^a_{-2} is 6×10^5 M^{-1} s^{-1} which is about one-third the value of the apparent second-order rate constant, k^a_4, of actin binding to the myosin product state (M**·ADP·P_i). The determination of k^a_{-2} allows a lower limit of 6 s^{-1} to be placed upon the first-order rate of ATP release from AM·ATP. This is to be compared with a value of $\leq 1.5 \times 10^{-4}$ s^{-1} for the equivalent steps of the myosin scheme; thus actin enhances the rate by a factor of 4×10^4 or more. A greater proportion of the bound ATP is released to the medium as ATP with increasing actin concentration. This reflects the contribution to rate limitation at saturating actin concentration of steps between myosin states dissociated from actin.

The actomyosin ATPase scheme prior to the steps of product release is understood qualitatively, in that actomyosin binds ATP, actin dissociates, and hydrolysis (eq 1) takes place on myosin while dissociated from actin (Lymn & Taylor, 1971), but our quantitative understanding is less satisfactory.

$$\begin{array}{ccccc} M + ATP & \leftrightarrow & M^*\cdot ATP & \overset{3}{\leftrightarrow} & M^{**}\cdot ADP\cdot P_i \\ {\scriptstyle A}\updownarrow & & {\scriptstyle 2}\updownarrow{\scriptstyle A} & & {\scriptstyle A}\updownarrow{\scriptstyle 4} \\ AM + ATP & \overset{1}{\leftrightarrow} & AM\cdot ATP & AM\cdot ADP\cdot P_i & \overset{5}{\leftrightarrow} AM + prod \end{array} \quad (1)$$

The apparent second-order rate constant of ATP association with acto-S1 has been measured by the change in light scattering upon dissociation of actin. White & Taylor (1976) obtained a value of 4×10^6 M^{-1} s^{-1} (pH 7, 40 mM KCl) for this rate constant. The equilibrium constant of ATP binding to S1,[1] K_{ATP}, and that of actin binding to S1, K_A, have been measured, but the difficulties of measuring such tight binding constants leave some uncertainties. Recent experimental values for K_{ATP} vary from 10^{10} to 3×10^{11} M^{-1} (Wolcott & Boyer, 1975; Goody et al., 1977; Cardon & Boyer, 1978) and values of K_A from 2×10^7 to 10^8 M^{-1} (Margossian & Lowey, 1976; Marston & Weber, 1975). The overall equilibrium constant between AM and M*·ATP is thus 100 to 15000, but the equilibrium constants of the elementary steps 1 and 2 are unknown. The experiments described in this paper were directed toward measuring k_{-1} and K_2.

The rate of ATP binding to S1 is fast compared with the ATPase rate; thus if one adds a stoichiometric amount of [γ-^{32}P]ATP to S1, there is a time, about 2 s after addition, when essentially all the ATP is bound, but little product release has occurred. Bagshaw & Trentham (1973) exploited this situation in two classic experiments. They quenched such a mixture at 2 s and found that about 10% of the nucleotide was still in the form ATP. When they quenched the reaction at times later than 2 s, the amount of ATP present was found to decay at the steady-state ATPase rate. They showed that the ATP was enzyme bound by means of an ATP chase experiment. Instead of quenching with perchloric acid at 2 s, they added an excess of unlabeled ATP and allowed hydrolysis to proceed for 1 min before quenching in perchloric acid. At this point no [γ-^{32}P]ATP remained, which showed that it was all bound at the time of addition of unlabeled ATP, and that this bound ATP was not in equilibrium with medium ATP. They concluded that the ATP cleavage step was in equilibrium during hydrolysis and thus the relative amounts of bound ATP and bound products were a measure of the equilibrium constant of the cleavage step on the enzyme.

We studied the effect of the addition of actin to such an equilibrium mixture of M*·ATP and M**·ADP·P_i. Actin can bind to both intermediates; it can bind to M*·ATP promoting the release of ATP and it can bind to M**·ADP·P_i promoting the release of products. The ratio of nucleotide released as ATP to that released as products reflects the apparent second-order rate constants of actin binding to the states M*·ATP (k^a_{-2})[2] and M**·ADP·P_i (k^a_4). The latter rate is known from steady-state ATPase measurements, and thus the experiment allows measurement of the apparent second-order rate constant of actin binding to M*·ATP.

A second method, ATP ⇌ HOH exchange, was used to determine the effect of actin on ATP release from M*·ATP. ATP ⇌ HOH exchange is the exchange of oxygens between medium water and the γ-phosphate of medium ATP. Current evidence is consistent with ATP ⇌ HOH exchange arising from multiple reversal of the hydrolytic cleavage step (Bagshaw et al., 1975), the phosphate of the M**·ADP·P_i state being free to rotate such that the oxygens are equivalent (Sleep et al., 1978). An ATP molecule is bound; one oxygen atom is incorporated into the phosphate on cleavage; upon reversal there are three chances out of four of this oxygen being retained in the γ-phosphate of ATP. Release of this bound ATP to the

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[1] Abbreviations used: S1, myosin subfragment 1; Tris, tris(hydroxymethyl)aminomethane; Mes, 2-(*N*-morpholino)ethanesulfonic acid; AMPP(NH)P, adenosine 5′-(β,γ-imino)triphosphate.

[2] k^a_{-2} and k^a_4 correspond to $k_{-1}K_2$ and K_4k_5 in the scheme of eq 1. The addition of a refractory state does not change the interpretation of k^a_{-2}, although it does of k^a_4.

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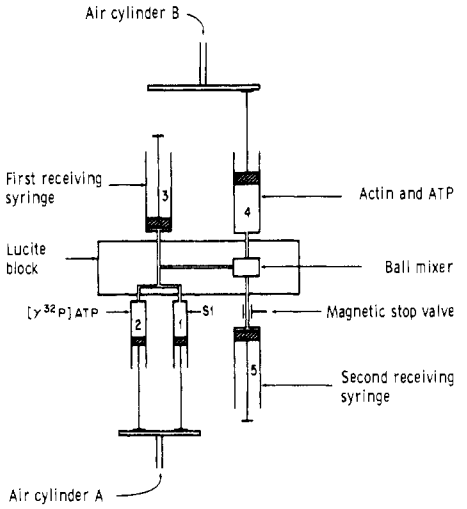


FIGURE 1: Quench flow apparatus.

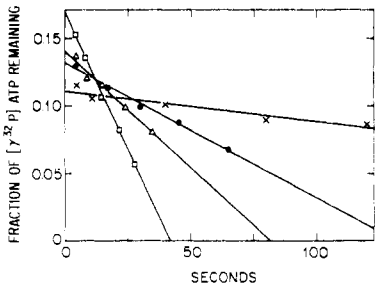


FIGURE 2: Time courses of quench flow experiments. S1(A1) (0.75 mL, 10 μM) was mixed with [γ-^{32}P]ATP (0.75 mL, 3 μM); after 2 s, 1.5 mL of actin and 2 mM MgATP were added. Reaction conditions: 5 mM KCl, 1 mM $MgCl_2$, 5 mM Tris-Mes (pH 7.0), 23 °C. Actin concentrations: (X) 0.05 mg/mL; (●) 0.1 mg/mL; (△) 0.2 mg/mL; (□) 0.5 mg/mL. Zero time corresponds to the time of addition of actin to M*·ATP.

medium results in ATP ⇌ HOH exchange. If more than one reversal occurs prior to the release of ATP, the extent of ATP ⇌ HOH exchange will be correspondingly greater. ATP ⇌ HOH exchange depends on more factors than the rate at which actin releases ATP from M*·ATP. However, a simultaneous measurement of the extent of ATP ⇌ HOH exchange and intermediate P_i ⇌ HOH oxygen exchange allows determination of the apparent second-order rate constant of actin binding to M*·ATP.

Materials and Methods

Proteins were prepared and their concentrations determined as described in Sleep & Boyer (1978). These experiments were done with the A1 fraction of S1 (Weeds & Taylor, 1975). Preparation of [γ-^{32}P]ATP was by the method of Schendel & Wells (1973).

Quench Flow Apparatus. For the experiment in which actin and cold ATP are added to the intermediate state, M*·ATP, the times between additions are almost within the range of hand mixing methods, but the quality of mixing required is much higher than might at first be apparent. At saturating actin concentrations the steady state rate of hydrolysis is about 14 s^{-1} (S1(A1), 23 °C) or a turnover time of 70 ms and so for a valid experiment the mixing of actin with M*·ATP must be complete in a time much less than 70 ms which requires the use of a quench flow apparatus (Figure 1).

Air cylinder A forces the contents of syringes 1, S1, and 2, [γ-^{32}P]ATP (1-mL syringes), via a T mixer into syringe 3. After a fixed delay from a delay solenoid, air cylinder B fires and simultaneously the stop valve opens and the contents of syringes 3, M*·ATP, and 4, actin and unlabeled ATP (3-mL syringes), are forced via a ball mixer (Berger et al., 1968) into syringe 5 (3-mL syringe) which is removed and portions of the contents are quenched in perchloric acid at a series of times. The sample tubes are then centrifuged, the supernatants neutralized with potassium hydroxide to precipitate potassium perchlorate, and the supernatants of this step applied to a column (0.7 × 3 cm) of anion-exchange resin (Bio-Rad AG1-X4, 200–400 mesh). Phosphate was eluted with 18 mL of 30 mM HCl, and ATP with 9 mL of 1 M HCl. Both phosphate and ATP fractions were counted to determine the fraction of the total counts in the form of ATP.

ATP ⇌ HOH and Intermediate P_i ⇌ HOH Exchange. The ATP ⇌ HOH exchange of acto-S1 was measured by hydrolysing 75–80% of a 2 mM ATP pool in ^{18}O-enriched water (2.85 atom % excess). The reaction was quenched in perchloric acid and the protein precipitate removed by centrifugation. The supernatant was neutralized with KOH and the supernatant of this step was applied to an anion-exchange column (3 × 0.7 cm, Bio-Rad, AG 1-X4, 200–400 mesh). Phosphate was eluted with 5 mL of 60 mM HCl, a further 10 mL of 60 mM HCl was used to elute ADP. The ATP fraction was then eluted in 5 mL of 1 M HCl. Charcoal was added to the phosphate fraction to remove any remaining ADP and the phosphate purified for ^{18}O analysis as described in the acompanying paper to determine intermediate P_i ⇌ HOH exchange. The ATP fraction was hydrolyzed in 1 M HCl for 45 min at 100 °C to release the β- and γ-phosphates which were purified and the ^{18}O content measured to determine ATP ⇌ HOH exchange.

Results

Effect of Actin on Release of Bound ATP. A substoichiometric amount of [γ-^{32}P]ATP is added to S1 and after 2 s 25% of the added nucleotide is in the form M*·ATP. At this time actin and unlabeled ATP are added and a fraction of the bound ATP is released into the unlabeled ATP pool. The pool then undergoes extensive hydrolysis by the acto-S1 ATPase. Time courses of such an experiment at 4 actin concentrations are shown in Figure 2. To avoid overcrowding the figure, the higher actin concentrations are not included. The ordinate is the fraction of [γ-^{32}P]ATP that remains at the time of perchloric acid quench. Zero time corresponds to the time of addition of actin and unlabeled ATP. The intercepts of these lines with the abscissa allow the steady-state rates of ATP hydrolysis at the various actin concentrations to be determined, and the expected hyperbolic dependence on actin concentration is observed.

The following observation confirms that [γ-^{32}P]ATP bound to S1 is released to the medium upon addition of actin: if the 2 mM ATP added with the actin is labeled with tritium, then the same rates of hydrolysis are obtained from the appearance of [^{3}H]ADP those derived from measurements with ^{32}P.

The most important information that can be derived from the data of Figure 2 is the values of the fraction of [γ-^{32}P]ATP remaining when data are extrapolated to zero time, the time of addition of actin and 2 mM unlabeled ATP. These extrapolated values are plotted against actin concentration in Figure 3. The amount of ATP released is expressed as the fraction of the bound ATP which was present at the time of actin addition, the latter figure being obtained from the perchloric acid quench part of the experiment. The main feature of the experimental points is the increase in the fraction of ATP released to the medium with increasing actin concentration; at high actin concentrations most of the bound ATP is released to the medium. The other important feature is that, upon extrapolation

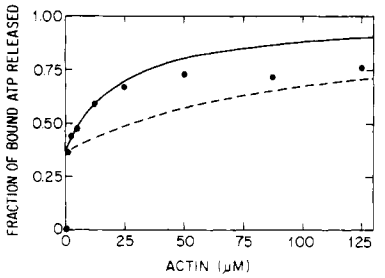


FIGURE 3: Plot of the fraction of bound [γ-^{32}P]ATP present at the time of actin addition which was released to the medium against actin concentration. Points derived from the data of Figure 2. The solid line is a theoretical curve (eq 7) with $r_0 = 0.36$ and $\gamma = 8 \times 10^{-6}$ M. The dashed line is a theoretical curve with $r_0 = 0.36$ and $\gamma = 3.6 \times 10^{-5}$ M. At the time of actin addition, 25% of the [γ-^{32}P] ATP was in the form of ATP bound to myosin.

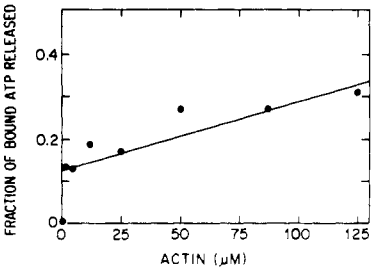


FIGURE 4: As Figure 3 but with 40 mM KCl. The solid line is a theoretical curve with $r_0 = 0.13$ and $\gamma = 5 \times 10^{-5}$ M.

to zero actin concentration, about one-third of the bound ATP is still released.

The main controls for these experiments are experiments of the type described by Bagshaw & Trentham (1973): first the 2-s perchloric acid quench already mentioned; second an unlabeled ATP chase experiment in the absence of actin. The chase experiments showed that 1–2% of the [γ-^{32}P]ATP remained unbound after 2 s. The time courses of Figure 2 are direct plots of the experimental data, while the small correction for unbound ATP has already been made in Figures 3 and 4.

The data will be dealt with in a qualitative manner in this section and the model, upon which the theoretical curves are based, will be treated in the Discussion section. At all actin concentrations, the amount of ATP released to the medium is a significant proportion of the ATP bound to S1 at the time of actin addition, which means that the apparent second order rate constant of actin binding to M*·ATP, as measured by the release of ATP, is about the same as the apparent second-order rate constant of actin binding to M**·ADP·P_i as measured by the release of products.

Another conclusion that qualitative considerations allow relates to the location of the rate-limiting step in the scheme. The rate of release of bound ATP is k_{-1} [AM·ATP] and that of products k_5[AM·ADP·P_i]. If the rate-limiting step of ATP hydrolysis of acto-S1 at saturating actin concentration were the release of products from AM·ADP·P_i, and the ATP cleavage step were fast such that the relative concentrations of M*·ATP and M*·ADP·P_i were governed by the equilibrium constant of the cleavage step, then [AM·ATP] = K_2 [AM·ADP·P_i]/K_3K_4 and the ratio of ATP released to products released would be $k_{-1}K_2/k_5K_3K_4$ which is not a function of actin concentration. The experimental results show a marked dependence upon actin concentration and thus the two states M*·ATP and M**·ADP·P_i are not in equilibrium during actin activated ATP hydrolysis. The origin of the actin dependence can also be considered in the following manner. The first step in releasing ATP as ATP is actin binding, whereas the first step toward its release as products is cleavage to M**·ADP·P_i, the rate of which is independent of actin concentration. Actin dependence of the proportion of bound ATP released as ATP will occur in the range of actin concentration at which the rate of actin binding to M*·ATP is comparable to the rate from M*·ATP to the M·ADP·P_i state to which actin recombines. The introduction of a refractory state to the scheme thus lowers the concentration range of actin dependence due to the rate from refractory to nonrefractory states reducing the overall rate from M*·ATP to the nonrefractory M·ADP·P_i state to which actin binds.

On extrapolation to zero actin concentration where the steady-state rate of ATP hydrolysis can be regarded as going to zero for the present purposes (ignoring the myosin ATPase rate), the cleavage step will approach equilibrium, and the ratio of bound ATP that is released to the medium as such, to that hydrolyzed before release, reflects the ratio of the apparent second-order rate constants of actin binding to the two states M*·ATP and M**·ADP·P_i. This extrapolated value corresponds to about 12% of the bound nucleotide (M*·ATP + M**·ADP·P_i) being released as ATP and 88% as products, which together with an equilibrium constant, K_3 of 2.2, gives a 1:3.5 ratio of the apparent rate constants ($k_{-1}K_2$:k_5K_4). In the Discussion section the shape of the curve is considered in more detail but the ratio of the apparent second-order rate constants is still governed by the amount of ATP released upon extrapolation to zero actin concentration. It is readily shown that the relationship given above is consistent with the general equation derived in the Appendix (eq 6) at zero actin concentration.

The data from similar experiments at moderate ionic strength (40 mM KCl) are shown in Figure 4. The results are qualitatively similar to those at low ionic strength, in that ATP release still shows a dependence upon actin concentration. However, at a given actin concentration a smaller proportion of the bound ATP is released to the medium and thus the ratio of the apparent second-order rate constant of actin binding to M*·ATP and to M**·ADP·P_i is correspondingly smaller than at 5 mM KCl.

The experiment was also performed with S1(A2) at low ionic strength (5 mM KCl). The results are qualitatively similar to those for the A1 fraction, but for a given actin concentration less ATP is released to the medium. A perchloric acid quench at 2 s showed that 0.26 of the added ATP was in the form M*·ATP. The proportion of ATP released to the medium as such is again quite dependent upon actin concentration; at 0.1 mg/mL, 13% of the bound ATP was released to the medium, while at 5 mg/mL 41% was released to the medium.

ATP ⇌ HOH Exchange. The experiments described are the first measure of the apparent second-order rate constant $k_{-1}K_2$ of actin binding to M*·ATP. To confirm the result, a second method seemed desirable. Measurement of oxygen exchange between medium ATP and medium water offered a second, quite separate approach. The first method is a transient method, in that initially there is an amount of M*·ATP and one determines in what form the bound ATP is released to the medium upon addition of actin. In contrast oxygen exchange is a steady-state method; oxygen atoms derived from medium water are being exchanged into the ATP pool at a constant rate as ATP is hydrolyzed.

The experiments described show that at low actin concentration about one-third of ATP bound in the form M*·ATP is released as such to the medium. At a low actin concentration

TABLE I: ATP ⇌ HOH Exchange of Acto-S1.[a]

[actin] μM	fraction of ATP hydrolyzed	intermediate exchange O/P	x	k/c	f	z	z'	pred a.p.e.	obsd a.p.e.
1	0.82	2.535	0.807	0.156	0.235	1.424	1.656	0.138	0.096
2	0.82	(2.034)	0.678	0.186	0.273	0.922	1.184	0.115	0.068
6	0.76	1.507	0.449	0.281	0.330	0.412	0.630	0.074	0.046

[a] A 2 mM MgATP pool was hydrolyzed in 2.85 atom % excess ^{18}O-enriched water. The reaction conditions were as for the quench flow experiments described in Figure 2 with a 4-mL reaction volume. At the three actin concentrations 1, 2, and 6 μM, the S1 concentrations were 0.5, 0.25, and 0.1 μM, respectively. Expressions for x, k, c, f, z, and z' are given in the Appendix. The O/P ratio at 2 μM actin is in brackets as the sample was lost in analysis. The figure is an interpolated value on the basis of the model for intermediate exchange given by Sleep & Boyer (1978).

intermediate oxygen exchange is high (the average number of oxygens from medium water which are present in a product phosphate molecule, the O/P ratio, is 2–3) and thus considerable reversal is occurring between the states M*·ATP and M**·ADP·P_i. ATP released will have incorporated oxygens from medium water and thus exchange of oxygens will occur between medium water and medium ATP. ATP ⇌ HOH exchange of myosin ATPase has been looked for before but not detected (Levy & Koshland, 1959); this is to be expected from the more recent measurements showing the very slow rate of release of ATP from M*·ATP in the absence of actin (Wolcott & Boyer, 1974; Mannherz et al., 1974).

ATP ⇌ HOH exchange has proved a powerful technique in, for example, assessing which steps require energy input in the synthesis of ATP by mitochondria (Rosing et al., 1977), but it has not previously been used to measure elementary rate constants; a quantitative treatment is given in the Appendix.

Qualitative considerations of the hydrolysis scheme (eq 2)

$$E + ATP \underset{k_{-1}}{\longleftrightarrow} E\cdot ATP \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} E\cdot ADP\cdot P_i \xrightarrow{k_3} E + \text{products} \qquad \text{HOH},\ \text{HOH} \tag{2}$$

allow one to deduce the most favorable conditions for observing ATP ⇌ HOH exchange. For appreciable oxygen from medium water to be incorporated into E·ATP, k_{-2} must be $\gtrsim k_3$ to allow significant reversal from E·ADP·P_i. These are the same conditions required for intermediate exchange. For acto-S1, k_3 is linearly dependent upon actin concentration and thus both intermediate exchange and ATP ⇌ HOH exchange decrease with increasing actin concentration. In the case of ATP ⇌ HOH exchange, increase in actin concentration has a small compensatory effect, for it increases the probability of ATP being released from E·ATP to the medium. The first effect of actin is dominant, and thus the extent of ATP ⇌ HOH exchange per ATP cleaved decreases with increasing actin concentration. The right-hand column of Table I shows that this prediction corresponds to our experimental observations. The quantitative treatment given in the Appendix shows that ATP ⇌ HOH depends on all four rate constants, k_{-1}, k_2, k_{-2}, and k_3. Intermediate exchange relates k_3 and k_{-2}; the equilibrium constant, K_2, relates k_2 and k_{-2}, which allows ATP ⇌ HOH exchange to relate k_{-1} and k_3, the same information given by the quench flow experiments described above.

Discussion

As a model for discussion we have used the Lymn–Taylor scheme in its most elementary form, with the additional assumption that steps 2 and 4, actin dissociation and rebinding, are in rapid equilibrium on the time scale of the first-order steps, k_{-1}, k_3, k_{-3}, and k_5.

$$\begin{array}{ccccc} & & M^*\cdot ATP & \overset{3}{\longleftrightarrow} & M^{**}\cdot ADP\cdot P_i \\ & & 2\downarrow\uparrow A & & A\downarrow\uparrow 4 \\ AM + ATP & \overset{1}{\longleftrightarrow} & AM\cdot ATP & & AM\cdot ADP\cdot P_i \overset{5}{\longleftrightarrow} AM + \text{products} \end{array} \tag{3}$$

Actin is added to an equilibrium mixture of M*·ATP and M**·ADP·P_i; the time dependence of the concentrations of the four species, AM·ATP, M*·ATP, M**·ADP·P_i and AM·ADP·P_i is of the form $Ae^{-\gamma_1 t} + Be^{-\gamma_2 t}$ + constant. The amount of ATP released to the medium is $\int_0^\infty k_{-1}$ [AM·ATP(t)]dt. A full treatment is given in the Appendix.

The fraction of the bound ATP released to the medium upon addition of actin is dependent on two variables, r_0 and γ (eq 6–8). The value of r_0 is simply the extrapolation of the experimental values to zero actin concentration. The theoretical curve plateaus at a value of 1.0, which corresponds to all the ATP being released, at infinite actin concentration and the value of γ determines the concentration range of the dependence. The experimental value of r_0, 0.36, in combination with the value for K_3, 2.2 under our experimental conditions, gives a ratio of 3.6 for $k_5K_4/k_{-1}K_2$ (eq 6).

The solid line in Figure 3 is a theoretical curve with $r_0 = 0.36$ and $\gamma = 8 \times 10^{-6}$ M. The fit is reasonable at low actin concentrations but is not good at high actin concentrations, for the experimental points plateau at about 75% of the bound ATP released and the model plateaus at 100%, whatever the values of the variable parameters. This point will be dealt with in more detail later. It is readily apparent that $\gamma = (k_3 + k_{-3})/K_4k_5$ is related to the contribution of ATP cleavage (step 3) to rate limitation of ATP hydrolysis. The value of 8×10^{-6} M is of the expected order of magnitude based on more direct measurements of $(k_3 + k_{-3})$ and K_4k_5 but the agreement is not good. The value of $(k_3 + k_{-3})$ is 80 s^{-1} under our experimental conditions (K. A. Johnson & E. W. Taylor, University of Chicago, personal communication). Our measurements of k' where $k'A$ is the steady-state ATPase rate at low actin concentration is 1.5×10^6 M^{-1} s^{-1} and this leads to a value of K_4k_5 of 2.2×10^6 M^{-1} s^{-1} when allowance is made for an equilibrium constant K_3 of 2.2. These values give an estimate for γ of 3.6×10^{-5} M, a factor of 4.5 greater than that used for the solid theoretical curve. The dashed line is a theoretical curve using this value of γ.

The data of an experiment at 40 mM KCl are shown in Figure 4. The proportion of ATP released to the medium extrapolated to zero actin concentration, r_0, is 0.13, which combined with an equilibrium constant K_3 of 2.2 gives a ratio

of $k_5K_4/k_{-1}K_2$ of 10.7 (eq 6), about two times greater than that obtained at 5 mM KCl. At 40 mM KCl k' is 2×10^5 M^{-1} s^{-1} which means that k_5K_4 is about 2.9×10^5 M^{-1} s^{-1} and thus $k_{-1}K_2$ is 2.7×10^4 M^{-1} s^{-1}. The proportion released is still significantly dependent upon actin concentration. The theoretical curve is based on a value of 5×10^{-5} M for γ (eq 7). At 40 mM KCl the proportion of nucleotide released as ATP is much smaller than at 5 mM KCl and the experimental errors are correspondingly greater but in all experiments the amount approximately doubled from the lowest to the highest actin concentration. The theoretical curve in Figure 4 is based on a value of 5×10^{-5} M for γ which is to be compared with the expected value of 5×10^{-3} M ($(k_3 + k_{-3}) = 130$ s^{-1}; K. Johnson & E. Taylor, personal communication). The difference between the value of γ to produce the best fit to our experimental data and the value from measurements of $(k_3 + k_{-3})$ and K_4k_5 is moderate at 5 mM KCl and large at 40 mM KCl.

This discrepancy should be considered alongside the problem of fully accounting for the results of Eisenberg & co-workers relating to the possible existence of a myosin product refractory state (Eisenberg & Kielley, 1972; Fraser et al., 1975; Chock et al., 1976). The recent observations that the rate of ATP cleavage is ionic strength dependent, being lower at low ionic strength, provide a partial but not complete explanation of their refractory state data; it is probable that this problem and the discrepancy in the expected and observed value of γ have a common explanation. The intermediate exchange data of Sleep & Boyer (1978) do not appear consistent with a refractory state of the form M**·ADP·P_i but as discussed in that paper a refractory state involving a phosphorylated intermediate or a metaphosphate such that hydrolysis occurs in the transition from refractory to nonrefractory states would be consistent with all the data.

It is important to note that, while the equations used to determine $k_{-1}K_2$ were formulated in terms of the simple Lymn–Taylor scheme, the measurement is equally valid for a refractory state scheme. If the extra step is designated x

$$\text{M**·ADP·P}_i\text{(R)} \underset{k_{-x}}{\overset{k_x}{\rightleftharpoons}} + \text{ M**·ADP·P}_i\text{(NR)}$$

then the proportion of bound ATP released on extrapolation to zero actin, r_0, which was $(1 + K_3)/(1 + k_5K_4K_3/k_{-1}K_2)$ (eq 6) becomes $(1 + K_3 + K_3K_x)/(1 + k_5K_4K_3K_x/k_{-1}K_2)$ but k_5K_4 which was $(V_m/K_m)(1 + K_3)/K_3$ becomes $(V_m/K_m)(1 + K_3 + K_3K_x)/K_3K_x$ which exactly compensates and thus as might be expected intuitively our measurement of $k_{-1}K_2$ is not changed by models with extra myosin product states.

We have determined that $k_{-1}K_2$ is about $2.2 \times 10^6/3.6 = 6 \times 10^5$ M^{-1} s^{-1} but the release of ATP from M*·ATP by actin gives no information about how this product should be split up. However, some limits can be placed upon these parameters because the value of K_2 is almost certainly less than $1/K_m$ or acto-S1 would be more associated than observed (Fraser et al., 1975) at actin concentrations which give steady-state ATPase rates close to V_m. The corresponding lower limit on k_{-1} is $6 \times 10^5/10^5 = 6$ s^{-1} and the true value is likely somewhat higher than this. The association of actin thus enhances the rate of ATP dissociation by a factor of $(\geq 6/\leq 1.5 \times 10^{-4})$ more than 4×10^4.

The proportion of the bound ATP released to the medium was found to plateau at 0.75 and not 1.0 as predicted from the simple model used. The model predicts a plateau at 1.0 because at saturating actin concentrations only the associated states are populated

$$\text{AM} + \text{ATP} \underset{k_{-1}}{\rightleftharpoons} \text{AM·ATP} \overset{k_d}{\rightleftharpoons} \text{AM·ADP·P}_i \rightleftharpoons \text{AM} + \text{Pr}$$

If $k_d = 0$, then, upon addition of saturating actin, bound ATP is trapped in the state AM·ATP until released to the medium as ATP. If $k_d \neq 0$ there is a competition between release (k_{-1}) and hydrolysis (k_d) and not all the bound ATP will be released as such. The position of the plateau can thus give some information about the contribution of the nondissociating pathway, which is useful for it is important to know whether the nondissociating pathway is significant in muscle. Taylor (1978) gave the formula for the ratio of the flux of the dissociating to that of the nondissociating pathway $R = k_3/k_dK_2[\text{A}]$. At a sufficiently high actin concentration the nondissociating pathway will dominate but it is necessary to establish at what actin concentration this will occur. This actin concentration is dependent on whether the model includes a refractory state for the following reason. To fit the data with a simple Lymn–Taylor scheme, the rate k_d is made sufficiently large to reduce the proportion of ATP released at infinite actin concentration from 1.0 to 0.75. For a refractory state model in the absence of a nondissociating pathway, the plot will plateau at a value of greater than 1.0 because actin can pull back M**·ADP·P_i(R) as well as M*·ATP to release ATP. The plateau thus requires lowering, not from 1.0 to 0.75 but from some higher value, the exact number depending on the rate constants used for the refractory state model; this necessitates a larger contribution from the nondissociating pathway. Use of eq 10 suggests that for a Lymn–Taylor scheme the fluxes of the two pathways would become equal only at a very high actin concentration (10–30 times the K_m for actin) but for a model involving a refractory state the actin concentration required might be significantly lower. The most important question is whether the nondissociating pathway is important in muscle and this is considered in the previous paper (Sleep & Boyer, 1978).

These experiments have a similarity to one reported by Sleep & Taylor (1976), although they have a different objective. In that experiment [γ-^{32}P]ATP was added to acto-HMM and, after dissociation was essentially complete but before significant hydrolysis had occurred, unlabeled ATP was added. It was found that essentially all the bound ATP was cleaved. When we add actin to M*·ATP, we find that some ATP is released and thus there might appear to be a significant discrepancy. This is not so for the following reasons. The experiment of Sleep & Taylor was done at low actin concentrations (30 μM) and at 50 mM KCl, conditions under which we observe only slight release (Figure 4). The second point is that, when we add actin in our experiment, M*·ATP and M**·ADP·P_i are in equilibrium and, at low actin concentration, competition is primarily between the second-order rate constants of actin binding to M*·ATP and M**·ADP·P_i. In the experiment of Sleep & Taylor, at the time of addition of cold ATP, most of the nucleotide is in the form M*·ATP, and thus initially competition is between actin binding to M*·ATP and hydrolysis. These two factors lead one to expect almost all the bound ATP to be hydrolysed as was observed.

Oxygen exchange processes which occur during ATP hydrolysis are moderately complex phenomena, and, while the current evidence is consistent with exchange being due to reversal of the hydrolytic cleavage step and free rotation of P_i, oxygen exchange cannot be regarded as a technique for measuring the rate constants of elementary steps with high accuracy.

We have used the relative values of the apparent second-order rate constants, k^a_{-2} and k^a_4, derived from the quench

flow experments, together with the measurements of intermediate exchange and the fraction of the ATP pool hydrolysed, to predict the extent of ATP ⇌ HOH exchange. Table I gives the data from the ATP ⇌ HOH exchange experiment together with the predicted values.

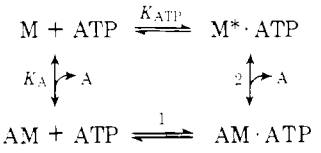
The calculation is separated into two parts: both the fraction, f (eq 13), of ATP molecules which have undergone exchange and the average extent of exchange, z (eq 12), when it occurs, must be calculated. The number of medium water oxygens per ATP is fz. The experimentally observed value for the atom percent excess ^{18}O in the P_i derived from acid hydrolysis of the ATP is ($f \times z \times$ atom percent excess of water)/8. The division by 8 arises from the fact that both β- and γ-phosphates are released upon acid hydrolysis of ATP. The theory is given in the Appendix.

The value of f is correct for the fraction exchanged but some ATP molecules have exchanged more than once and thus the predicted value has to be increased. A crude correction can be made on the basis that of the fraction f of the ATP pool which has undergone exchange, the fraction which has double exchanged is roughly $f^2/2$, and thus f should be corrected to ($f + (f^2/2)$). In practice the second exchange will be somewhat less productive for there is a possibility of exchanging out those oxygens which have already been derived from water in the first exchange. The corrections in Table I are a result of a proper analysis of the problem (z being replaced by z'), but they are of a size suggested by the crude approach outlined above.

The experimental values are 60–70% of the predicted values, but, considering the complexity of the phenomenon of ATP ⇌ HOH exchange, they are in good agreement. It shows that the values derived from the quench flow experiment are essentially correct, and that our understanding of ATP ⇌ HOH exchange is satisfactory.

In principle, measurement of ATP ⇌ HOH exchange, intermediate P_i ⇌ HOH exchange and the ratio k^a_{-2}/k^a_4 allows one to assess the plausibility of a model in which hydrolysis occurs in the step from the refractory to nonrefractory states (a possibility raised by Sleep & Boyer, 1978). For such a model ATP ⇌ HOH exchange would be less than for models in which hydrolysis were the first step after dissociation of AM·ATP, but, while the discrepancy is in this direction, it is too small to allow any firm conclusions on this point.

Thermodynamics of the Steps of the Actomyosin Scheme Prior to Hydrolysis. It is of interest to see whether the measured rate and equilibrium constants of that part of the scheme prior to the cleavage of ATP form a consistent thermodynamic scheme.



(4)

A difficulty in assessing the consistency of this scheme is that association of actin and S1 is highly ionic strength dependent and the various measurements have been made under different conditions. Also, as mentioned earlier, some uncertainties remain about the values of the equilibrium constants of ATP and actin binding to S1.

The best method of measuring the binding constant of actin to S1 (K_A) is that of Greene & Eisenberg (1978) who analyzed the equivalent thermodynamic box to eq 4 for the nucleotide AMPP(NH)P. They only made the measurement at high ionic strength ($I = 0.14$) and obtained the value of 2×10^7 M^{-1} in agreement with Marston & Weber (1975). We use the values of Marston & Weber at low ionic strength for these calculations.

The equilibrium constant of ATP binding to S1 (K_{ATP}) as determined by two methods has been reported to be 10^{10} to 3×10^{11} M^{-1} (Wolcott & Boyer, 1975; Goody et al., 1977). Part of the apparent discrepancy is the result of different experimental conditions. Cardon & Boyer (1978) have looked at the problem by both methods under the same experimental conditions (pH 7) and the ATP binding constant is in the range $1\text{–}3 \times 10^{10}$ M^{-1}.

First we will consider the thermodynamics at 40 mM KCl. The apparent second-order rate constant of ATP binding to acto-S1 is 4×10^6 M^{-1} (White & Taylor, 1976) as determined from the rate of acto-S1 dissociation by light scattering. The simplest interpretation of this result is that it measures k_{+1}. The apparent second-order rate constant of actin binding to M*·ATP is 2.7×10^4 M^{-1} s^{-1} as determined in this paper, and the simplest interpretation is that this is $k_{-1}K_2$. In combination these numbers give a value of 150 for K_1/K_2. With use of the Marston–Weber value for K_A of 10^8 M^{-1} and a value of $1\text{–}3 \times 10^{10}$ M^{-1} for K_{ATP}, the equilibrium between AM and M*·ATP derived from equilibrium constants via the myosin pathway is 100–300. This is in good agreement with the value of 150 from kinetic constants via AM·ATP. A similar calculation can be performed at 5 mM KCl. The thermodynamic estimate remains the same at 100–300. The value of k_1 is about 8×10^6 M^{-1} s^{-1} by extrapolation of the results of White & Taylor (1976), and our value of $k_{-1}K_2$ is 6×10^5 M^{-1} s^{-1} and the combination of these numbers gives an equilibrium constant of 13 between AM and M*·ATP. The agreement at this ionic strength is not as good but this probably arises in part from the value of K_A used. Marston & Weber reported significant variability between experiments but their average value at both 10 and 40 mM KCl was about 10^8 M^{-1}. Quantities related to the binding of actin to S1 states which are more readily measurable, such as the steady state ATPase, k_m for actin, continue to show a marked ionic strength dependence down to a very low ionic strength and the acto-S1 binding constant would be expected to follow this trend.

The value determined for the apparent second-order rate constant of actin binding to M*·ATP by quench flow methods is thus in good agreement with the value derived from measurement of ATP ⇌ HOH oxygen exchange and is reasonably consistent with previously determined equilibrium and rate constants.

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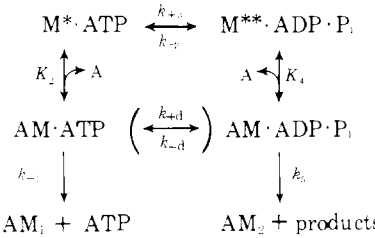
Appendix

Kinetics of the Quench Flow Experiments:



(5)

Steps 2 and 4 are assumed to be in rapid equilibrium; let X = [AM·ATP] + [M*·ATP] and Y = [AM·ADP·P_i] + [M**·

ADP·P_i]. The basic equations are thus:

$$\frac{d[AM_1]}{dt} = aX \qquad \text{where} \quad a = k_{-1}\frac{K_2[A]}{(1 + K_2[A])}$$

$$\frac{dX}{dt} = -(a + f)X + cY \qquad h = k_5\frac{K_4[A]}{(1 + K_4[A])}$$

$$\frac{dY}{dt} = fX - (c + h)Y \qquad f = \frac{k_3}{(1 + K_2[A])}$$

$$\frac{d[AM_2]}{dt} = hY \qquad c = \frac{k_{-3}}{(1 + K_4[A])}$$

The solution is of the form: $X = \alpha e^{\lambda_1 t} + \beta e^{\lambda_2 t} + \text{constant}$ where

$$\lambda_1 + \lambda_2 = -(a + c + f + h)$$

$$\lambda_1\lambda_2 = ac + ah + fh$$

The boundary conditions are:

At $t = 0$ $\quad X = X_0 = \alpha + \beta + \text{constant}$

At $t = \infty$ $\quad X = 0$, therefore constant = 0

At $t = 0$ $\quad \dot{X} = -(f + a)X_0 + cY_0 = \lambda_1\alpha + \lambda_2\beta$

$$\beta = \frac{-[cY_0 - X_0(f + a + \lambda_1)]}{(\lambda_1 - \lambda_2)}$$

$$\text{ATP released} = \int_0^\infty aX(t)dt = -a\left(\frac{\alpha}{\lambda_1} + \frac{\beta}{\lambda_2}\right)$$

$$\text{let } r = \frac{\text{ATP released}}{X_0}$$

$$= \frac{(1 + K_3 + k_5K_4[A]/k_{-3})}{(1 + k_5K_4K_3/k_{-1}K_2 + k_5K_4[A]/k_{-3})}$$

At zero actin concentration

$$r_0 = \frac{1 + K_3}{(1 + k_5K_4K_3/k_{-1}K_2)} \tag{6}$$

$$r = \frac{(1 + K_3 + k_5K_4[A]/k_{-3})}{(1 + K_3)/r_0 + k_5K_4A/k_{-3}}$$

$$r = \frac{(\gamma + [A])}{(\gamma/r_0 + [A])} \tag{7}$$

$$\text{where } \gamma = (k_3 + k_{-3})/K_4k_5 \tag{8}$$

Corresponding steady-state ATPase rate

$$v = \frac{k_5K_4[A]}{\frac{1 + K_3}{K_3} + \left(1 + \frac{k_5}{k_3} + \frac{K_2}{K_4K_3}\right)K_4[A] + \frac{k_5}{k_3}K_2K_4[A]^2} \tag{9}$$

The derivation can readily be extended to include the effects of step

$$AM{\cdot}ATP \underset{k_{-d}}{\overset{k_d}{\rightleftharpoons}} AM{\cdot}ADP{\cdot}P_i$$

$$r = \frac{(1 + K_3)(1 + k_{-d}K_4[A]/k_{-3}) + k_5K_4[A]/k_{-3}}{\{1 + k_5K_4(K_3 + k_dK_2[A]/k_{-3})/k_{-1}K_2 + (k_5 + k_{-d})K_4[A]/k_{-3}\}}$$

At infinite actin concentration this reduces to

$$r_\infty = \frac{(1 + K_3)k_{-d} + k_5}{\frac{k_5k_d}{k_{-1}} + k_5 + k_{-d}}$$

Neglecting terms in k_{-d}

$$k_d = k_{-1}(1/r_\infty - 1) \tag{10}$$

ATP ⇌ HOH Exchange. As was discussed in more detail by Sleep & Boyer (1978), all the current evidence is consistent with the oxygen exchange phenomena associated with ATP hydrolysis by acto-S1, being due to the reversal of the hydrolytic cleavage step. On the basis of similar assumptions to the case of intermediate exchange, regarding freedom of P_i rotation such that the oxygens are equivalent and rate limitation being due to the rate of reversal of the hydrolytic cleavage step, the extent of ATP ⇌ HOH exchange can be predicted from the elementary rate constants of the scheme.

The calculation of the expected ATP ⇌ HOH exchange falls into two parts. First, what is the average number of medium water oxygens, z, in the γ-phosphate of an ATP which has undergone exchange? Second, when a given fraction of the ATP pool has been hydrolyzed, what fraction, f, of the remaining pool has undergone exchange? The predicted number of atoms of oxygen from medium water per ATP molecule is thus fz.

The Average Number of Medium Water Oxygen Atoms per ATP Molecule Released to the Medium:

$$z = \sum_0^\infty p(n)O(n) / \sum_0^\infty p(n)$$

where $p(n)$ is the probability of release of ATP into the medium after n reversals of the hydrolysis step.

$O(n)$ is the number of oxygen atoms derived from medium water in the ATP released after n such reversals.

$$M + ATP \underset{k_{-1}}{\rightleftharpoons} M^*{\cdot}ATP \underset{HOH}{\overset{HOH}{\underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}}}} M^{**}{\cdot}ADP{\cdot}P_i \overset{k_3}{\rightleftharpoons} M + \text{prod} \tag{11}$$

Consider an ATP molecule which has bound to S1, forming the state M*·ATP. The probability of release of ATP to the medium is

$$y = k_{-1}/(k_{-1} + k_2)$$

The probability of the ATP being hydrolyzed to form M**·ADP·P_i, reversal to M*·ATP, and being released to the medium as ATP is $p(1) = (1 - y)xy$, where

$$x = k_{-2}/(k_{-2} + k_3)$$

The average number of water oxygens in the γ-phosphate of this ATP, ($O(1)$), will be 3/4. For each successive reversal, the probability of going from M*·ATP to M**·ADP·P_i is $(1 - y)$, and the probability of returning to M*·ATP is x. Thus $p(n) = y[(1 - y)x]^n$; correspondingly $O(n) = \Sigma_{i=1}^n (3/4)^i$ (Wolcott & Boyer, 1975).

The infinite series for z has an analytic solution (eq 12).

$$z = \frac{\sum_1^\infty y[(1 - y)x]^n \sum_{i=1}^n \left(\frac{3}{4}\right)^i}{\sum_0^\infty y[(1 - y)x]^n}$$

$$= [1 - (1 - y)x] \sum_1^\infty [(1 - y)x]^n \left(\frac{1 - \left(\frac{3}{4}\right)^{n+1}}{1 - \frac{3}{4}} - 1\right) =$$

$$4[1-(1-y)x]\left(\frac{3}{4}\sum_{0}^{\infty}[(1-y)x]^n - \frac{3}{4}\sum_{1}^{\infty}\left[\frac{3}{4}(1-y)x\right]^n\right)$$

$$= 3[1-(1-y)x]\left([1-(1-y)x]^{-1} - 1 - \left(1-\frac{3}{4}(1-y)x\right)^{-1} + 1\right) = \frac{3}{4}\frac{(1-y)x}{1-\frac{3}{4}(1-y)x} \quad (12)$$

The value of x is related to the extent of intermediate exchange, ($O/P = 4/(1 + 3(1 - x))$; Sleep & Boyer, 1978). The value of $y = k_{-1}K_2A/(k_{-1}K_2[A] + k_3)$ can be expressed in terms of x for the quench flow experiments measured the ratio $k_5K_4/k_{-1}K_2$ as 3.6 and $k_3 = K_3k_{-3}$.

The Proportion of the ATP Pool Which Has Bound to S1 and Returned to the Pool as a Function of the Extent of Hydrolysis of the ATP Pool. Let w = number of ATP molecules per S1 molecule (w_0 at $t = 0$); x = the number of ATP molecules per S1 molecule which have bound to S1 and returned to the pool; v = the rate of release (s^{-1}) of products to the medium from M*·ATP; k = the rate of release (s^{-1}) of ATP to the medium from M*·ATP; g = fraction of S1 in the state M*·ATP.

$$\frac{dw}{dt} = -vg$$

therefore

$$w = w_0 - vgt$$

$$\frac{dx}{dt} = -(vx/w - k(w-x)/w)g$$

Let the fraction of ATP pool which has undergone exchange be $f = x/w$

$$\frac{dx}{dt} = -[vf - k(1-f)]g$$

$$\frac{dx}{dt} = \frac{df}{dt}(w_0 - vgt) - fvg = -[vf - k(1-f)]g$$

$$\int_0^f \frac{df}{kg(1-f)} = \int_0^t \frac{dt}{w_0 - vgt}$$

$$f = 1 - \exp[k/v \ln(\text{fraction of ATP pool remaining})] \quad (13)$$

The term k/v in eq 13 needs expression in terms of measurable parameters.

$$k = k_{-1}K_2[A]$$

$$v = (k_3 - k_{-3}[M^{**}\cdot ADP\cdot P_i]/[M^*\cdot ATP])$$

where [M*·ATP] and [M**·ADP·P_i] are the steady-state concentrations.

$$[M^{**}\cdot ADP\cdot P_i] = \frac{k_3}{(k_{-3} + k_5K_4[A])}[M^*\cdot ATP]$$

$$k/v = k_{-1}K_2[A]/(k_3 - k_{-3}k_3/(k_{-3} + k_5K_4[A]))$$

$$k/v = \left[\frac{K_3k_{-3}}{k_{-1}K_2[A]}\left(1 - \frac{1}{1 + \frac{k_5K_4[A]}{k_{-3}}}\right)\right]^{-1} \quad (14)$$

K_3 is measured directly as 2.2; $k_{-1}K_2$ can be expressed as $k_5K_4/3.6$ from the quench flow experiments; $k_5K_4[A]/k_{-3}$ is directly related to intermediate exchange for $O/P = 4/(1 + 3(1 - x))$ where $x = k_{-3}/(k_{-3} + k_5K_4[A])$ (Sleep & Boyer, 1978).

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