Exchange between Inorganic Phosphate and Adenosine 5′-Triphosphate in the Medium by Actomyosin Subfragment 1†

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ABSTRACT: The ATP ⇌ P_i exchange of actin-activated myosin subfragment 1 was investigated as a function of actin concentration. The maximum rate of exchange was 1.7×10^{-3} s^{-1} at a P_i concentration of 2 mM, and this rate was independent of medium ADP concentration. The observed rate is 50 times faster than expected if reversal starts from the AM·ADP state that can be formed from medium ADP. The existence of a preceding higher energy AM′·ADP state which exists in significant concentration only during the steady-state hydrolysis of ATP accounts for this discrepancy and also for the independence of the rate of ATP ⇌ P_i exchange on medium ADP concentration. Addition of ADP and P_i to myosin results in the formation of an equilibrium amount of enzyme-bound ATP. The inhibitory effect of actin on the formation of enzyme-bound ATP has been investigated and a value of 4×10^6 M^{-1} has been determined for the binding constant of actin to M*·ADP and an upper limit of 2×10^4 M^{-1} has been determined for the binding constant of actin to M*·ATP.

The major steps toward understanding the kinetics of acto-S1 ATPase[1] were made by the use of transient kinetic methods (Lymn & Taylor, 1971; Chock et al., 1976), but it is more difficult to apply such methods to the energy transducing ATPase of muscle fibers. These circumstances increase the importance of more indirect, steady-state methods such as ATP ⇌ P_i exchange. This method has been used to investigate the mechanochemistry of muscle fibers (Gillis & Marechal, 1974; Ulbrich & Ruegg, 1976, 1977), but in isolation the data are difficult to interpret and the need to study the exchange of simpler systems is indicated. Measurements of the ATP ⇌ P_i exchange of actomyosin have been reported (Wolcott & Boyer, 1974; Paulsen, 1976), but actomyosin cannot be regarded as a simple system due to the unknown effects of steric and mechanical constraints arising from both actin and myosin being in the form of filaments. In this paper we report an investigation of the better understood acto-S1 system.

The dependence of acto-S1 ATP ⇌ P_i exchange on actin concentration agrees reasonably well with the theoretical curves based on a simple Lymn–Taylor scheme, but this observation in itself does not increase our understanding of the kinetics of acto-S1. The method in isolation lacks power due to the number of unknown parameters on which the rate of exchange is dependent. In the case of myosin (Wolcott & Boyer, 1974) a burst of [γ-^{32}P]ATP formation was found, corresponding to the formation of bound ATP, as well as a rate of ATP ⇌ P_i exchange. In combination the burst and rate allowed the first-order rate constant of ATP release to be determined. For acto-S1 the logic can be inverted; the rate of release of bound ATP has been determined by a separate method (Sleep & Hutton, 1978), and thus the rate of ATP ⇌ P_i exchange allows the concentration of bound [γ-^{32}P]ATP to be determined, a parameter of relevance to the product-release steps of the acto-S1 mechanism.

The measurement of the rate of release of ATP from S1 by actin (Sleep & Hutton, 1978) involved the state M*·ATP formed from medium ATP. Before using this rate to interpret ATP ⇌ P_i exchange experiments, it is desirable to show that the bound ATP species formed from ADP and P_i has the same properties as that formed from ATP. This point has a more fundamental relevance to the acto-S1 scheme because it was an assumption involved in both methods of determining the ATP binding constant of S1 (Wolcott & Boyer, 1974; Mannherz et al., 1974; Cardon & Boyer, 1978).

Experimental Procedure

Myosin was prepared from rabbit skeletal muscle by the method of Perry (1955). Myosin subfragment 1 (S1) was made and the A1 fraction was separated by the method of Weeds & Taylor (1975). Actin was prepared by the method of Drabikowski & Gergely (1964). Preparation of [γ-^{32}P]ATP was essentially by the method of Penefsky et al. (1960).

ATP ⇌ P_i Exchange. Low ionic strengths were used for these experiments to allow use of actin concentrations far above the K_m for the ATPase. The acto-S1 reaction solutions at the various actin concentrations were prepared, and an aliquot of each was used to determine the steady-state ATPase rate which was measured by using [γ-^{32}P]ATP, separation of ATP and P_i being by charcoal adsorption. A second aliquot of the acto-S1 reaction solution was used to measure ATP ⇌ P_i exchange. The reaction was started by the addition of [^{3}H]ATP and $^{32}P_i$, and samples were quenched in a cold solution of 0.5 M perchloric acid and 2 mM EDTA and analyzed for [γ-^{32}P]ATP/$^{32}P_i$ and [^{3}H]ATP/[^{3}H]ADP ratios. The method of separation of ATP, ADP, and P_i was essentially that of Smith et al. (1976): a charcoal adsorption followed by the use of an anion-exchange column (Bio-Rad AG1-X4, 200–400 mesh). The charcoal step was modified to a column procedure which gave somewhat better separation and also allowed simultaneous processing of 30 samples. The acid-quenched samples were applied to charcoal columns (75 mg of charcoal on a Celite support in a 7-mm diameter column). The columns then received a wash with 2 mL of water, two washes with

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[1] Abbreviations used: S1, myosin subfragment 1; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid.

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5 mL of 0.3 M perchloric acid, 0.1 M phosphate, and 0.025 M pyrophosphate, and a further wash with 5 mL of water. Nucleotides were then eluted with 5 mL of 95% ethanol–1 M NH_3 (40:60 v/v) and applied to anion-exchange columns as before. Suction was used to accelerate the flow rate through the charcoal columns. The ATP fraction from the anion-exchange column was counted for ^{32}P by using Cerenkov radiation; after counting the recovery of ATP was determined spectrophotometrically and was typically 60%. An aliquot of the washings was used to determine the total ^{32}P counts in the sample. In all calculations the ratio of [γ-^{32}P]ATP to $^{32}P_i$ was used and thus the results were unaffected by any variation in sample size. Aliquots of the ATP and ADP fractions were counted for ^3H to give the [^{3}H]ATP/[^{3}H]ADP ratio as a check of the steady-state ATPase rates.

Release of ATP from S1 by Actin. Labeled P_i was added to a small volume of S1, ADP, and unlabeled P_i, and after waiting 1 min to allow an equilibrium amount of myosin-bound ATP to form, two aliquots were quenched to determine the amount of bound ATP formed. The major aliquot was then injected into a tube of actin and unlabeled ATP which was being stirred with a magnetic flea. Aliquots of the reaction mixture were quenched, and the ATP and P_i were separated as before. In this case the carrier ATP was labeled with ^3H to determine the recovery. The analogous [γ-^{32}P]ATP experiment was done in a similar manner; a substoichiometric amount of [γ-^{32}P]ATP was added to a small stirred volume of S1, followed 3 s later by a solution of actin and unlabeled ATP. Two controls were performed, a perchloric acid quench and an unlabeled ATP chase (Bagshaw & Trentham, 1973), to determine the amount of bound and free [γ-^{32}P]ATP at 3 s. For the experiments involving the use of [γ-^{32}P]ATP, sufficient separation was achieved without the use of the charcoal column step. The sample was quenched as before and 1 μmol each of carrier ATP and P_i was added. After centrifugation the supernatant was neutralized with cold KOH to precipitate potassium perchlorate. The sample was centrifuged again and the supernatant was applied to an anion-exchange column. The ratio of counts in ATP to counts in P_i was determined.

Theory. The derivation for the dependence of ATP $\rightleftharpoons$ P_i exchange on actin concentration is given for a simple Lymn–Taylor scheme (eq 1), without a refractory state (Chock et al., 1976) or a contribution from a nondissociating pathway (AM·ATP $\rightleftharpoons$ AM·ADP·P_i).

$$\begin{aligned} &\mathrm{AM} + \mathrm{ATP} \overset{1}{\rightleftharpoons} \mathrm{AM{\cdot}ATP} \overset{A}{\underset{2}{\rightleftharpoons}} \mathrm{M^{*}{\cdot}ATP} \overset{3}{\rightleftharpoons} \mathrm{M^{**}{\cdot}ADP{\cdot}P_i} \overset{A}{\underset{4}{\rightleftharpoons}} \\ &\mathrm{AM{\cdot}ADP{\cdot}P_i} \overset{5}{\underset{P_i}{\rightleftharpoons}} \mathrm{AM'{\cdot}ADP} \overset{6}{\rightleftharpoons} \mathrm{AM} + \mathrm{ADP} \quad (1) \end{aligned}$$

Steps 2 and 4 are assumed to be fast equilibria. The addition of a dagger symbol to a state signifies that it contains medium P_i. A prime symbol has been placed on AM·ADP (AM′·AMP), and this is used throughout to refer to the AM·ADP state to which P_i binds. We will discuss whether AM′·ADP is the same as the state (AM·ADP) formed from medium ADP. The equilibrium constants K_i are equal to k_i/k_{-i} except for K_2 which equals k_{-2}/k_2. (It is convenient to have the same dimensions for equilibrium constants K_2 and K_4 describing steps in which actin associates with myosin states.) Following standard usage for acto-S1 kinetics, the steady-state ATPase rate v, the ATPase rate at saturating actin concentration V_{max}, and the rate of ATP $\rightleftharpoons$ P_i exchange V_{ex} all have the dimensions of s^{-1}, i.e., the conventional rate has been divided by the S1 concentration.

Let

$$X = [\mathrm{AM{\cdot}ATP^{\dagger}}] + [\mathrm{M^{*}{\cdot}ATP^{\dagger}}]$$

$$Y = [\mathrm{AM{\cdot}ADP{\cdot}P_i^{\dagger}}] + [\mathrm{M^{**}{\cdot}ADP{\cdot}P_i^{\dagger}}]$$

The equations describing exchange during the steady-state are

$$\frac{dY}{dt} = 0 = k_{-5}[\mathrm{AM'{\cdot}ADP}][P_i] - \frac{k_5K_4[A]Y}{1 + K_4[A]} + \frac{k_3X}{1 + K_2[A]} - \frac{k_{-3}Y}{1 + K_4[A]} \quad (2)$$

$$\frac{dX}{dt} = 0 = \frac{k_{-3}Y}{1 + K_4[A]} - \frac{k_{-1}K_2[A]X}{1 + K_2[A]} - \frac{k_3X}{1 + K_2[A]} \quad (3)$$

From eq 3

$$Y = X\left(\frac{k_{-1}K_2[A] + k_3}{1 + K_2[A]}\right)\left(\frac{1 + K_4[A]}{k_{-3}}\right) \quad (4)$$

From eq 2 and 4

$$X = \frac{k_{-5}[\mathrm{AM'{\cdot}ADP}][P_i](1 + K_2[A])}{(K_3k_5K_4 + k_{-1}K_2)[A] + k_{-1}K_2k_5K_4[A]^2/k_{-3}}$$

For the rate of ATP $\rightleftharpoons$ P_i exchange

$$V_{ex} = k_{-1}[\mathrm{AM{\cdot}ATP^{\dagger}}] = \frac{k_{-1}K_2[A]X}{1 + K_2[A]} = \frac{k_{-1}K_2k_{-5}[\mathrm{AM'{\cdot}ADP}][P_i]}{K_3k_5K_4 + k_{-1}K_2 + k_{-1}K_2k_5K_4[A]/k_{-3}}$$

Experimentally accessible concentrations of medium ADP were found to have no effect on the rate of acto-S1 ATP $\rightleftharpoons$ P_i exchange (see Table I). The state AM′·ADP is present because it is a steady-state intermediate and the release of ADP can be treated as irreversible.

$$[\mathrm{AM'{\cdot}ADP}]/M_0 = v/(v + k_6) \quad (5)$$

M_0 is the total S1 concentration. The rate k_6 is expected to be much greater than v; this is confirmed by the slow rate of ATP $\rightleftharpoons$ P_i exchange and thus

$$[\mathrm{AM'{\cdot}ADP}]/M_0 \approx \frac{v}{k_6} = \frac{V_{max}[A]}{k_6(K_m + [A])}$$

$$V_{ex} = \left(\frac{k_{-1}K_2k_{-5}[P_i]}{K_3k_5K_4 + k_{-1}K_2 + k_{-1}K_2k_5K_4[A]/k_{-3}}\right) \times \left(\frac{V_{max}[A]}{k_6(K_m + [A])}\right)$$

$$V_{ex} \propto \frac{[A]}{(K_m + [A])[[(K_3k_5K_4 + k_{-1}K_2)/(k_{-1}K_2k_5K_4)]k_{-3} + [A]]} \propto \frac{[A]}{(K_m + [A])(K_z + [A])} \quad (6)$$

where

$$K_z = \frac{K_3k_5K_4/(k_{-1}K_2) + 1}{k_5K_4}k_{-3} \quad (7)$$

Taking $k_{-3} = 90\ s^{-1}$ [extrapolation of the data of Johnson & Taylor (1978)], $K_3k_5K_4/(k_{-1}K_2) = 8$ (Sleep & Hutton, 1978), $k_5K_4 = V_m/K_m[(1 + K_3)/K_3] = (2.2 \times 10^6\ M^{-1}\ s^{-1})(3.2/2.2) = 3.2 \times 10^6\ M^{-1}\ s^{-1}$ (from data of Figure 2) and, hence, $K_z = 100\ \mu M$.

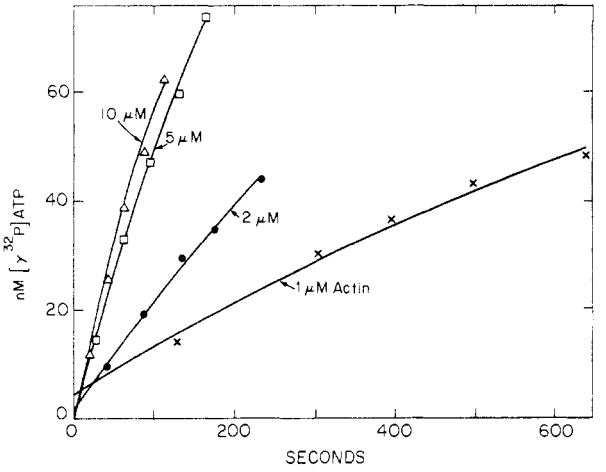


FIGURE 1: Time course of ATP ⇌ P_i exchange. The reaction was initiated by the addition of 50 μL of [^{3}H]ATP and $^{32}P_i$ to 250 μL of acto-S1, the final concentrations being 0.5 μM S1, actin as specified, 3 mM $MgCl_2$, 2 mM ATP, 10 mM Hepes, and 2 mM P_i, neutralized to pH 7 with Tris. Aliquots of 50 μL were quenched in 0.5 M perchloric acid and 2 mM EDTA at the times indicated and analyzed for [γ-^{32}P]ATP/$^{32}P_i$ and [^{3}H]ATP/[^{3}H]ADP ratios. The curves correspond to the equation $[[\gamma\text{-}^{32}P]ATP] = C + A(1 - t) \ln (1 - t)$ (eq 8).

Treatment of the ATP ⇌ P_i Exchange Data. The equation for the fraction of the ATP pool, f, which has undergone exchange was given by Sleep & Hutton (1978) when considering ATP ⇌ HOH exchange

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

where k is the rate of ATP ⇌ P_i exchange and v is the ATPase rate.

If the units of time are chosen such that $t = 1$ when hydrolysis is complete

$$[[\gamma\text{-}^{32}P]ATP] \propto (1 - t)[1 - (1 - t)^{k/v}]$$

but $k \ll v$; thus

$$[[\gamma\text{-}^{32}P]ATP] \propto (1 - t) \ln (1 - t)$$

The experimental curves are thus fitted to

$$[[\gamma\text{-}^{32}P]ATP] = A + C(1 - t) \ln (1 - t) \qquad (8)$$

The constant A allows for the possibility of a burst of enzyme-bound ATP being formed. Least-squares fitting to this equation is simple as its form is similar to $y = A + Cx$. The concentration of [γ-^{32}P]ATP reaches a maximum when a fraction exp(−1) of the ATP pool remains.

Results

ATP ⇌ P_i Exchange of Acto-S1. The results of an acto-S1 ATP ⇌ P_i exchange experiment are shown in Figure 1. Only the four lowest actin concentrations are shown to avoid overcrowding the figure. The rates of exchange derived from these data are plotted against actin concentration in Figure 2. Included in this figure is the steady-state ATPase rate as a function of actin concentration. These latter data were obtained by using aliquots of the protein solution prepared for the ATP ⇌ P_i exchange experiment, the rates being determined with [γ-^{32}P]ATP and charcoal adsorption. Similar results were obtained directly from the [^{3}H]ATP/[^{3}H]ADP ratio of the ATP ⇌ P_i exchange samples.

An ADP molecule is necessary to form ATP from medium P_i: this might be derived either from the medium or from an ATP which has been hydrolyzed. The effect of varying the

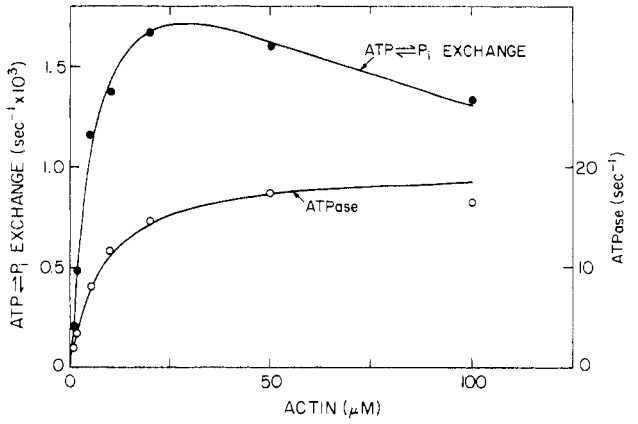


FIGURE 2: Dependence of the rate of ATP ⇌ P_i exchange and ATPase on actin concentration. The data are derived from the experiment of Figure 1. The curve for the ATPase (○) is a hyperbola with a K_m of 8 μM and V_{max} of 20 s^{-1}. The curve for the ATP ⇌ P_i exchange (●) corresponds to the equation $V_{ex} = J[A]/[(K_m + [A])(K_z + [A])]$, the values of K_m and K_z being 8 and 100 μM.

Table I: Effect of Medium ADP Concentration on the Rate of ATP ⇌ P_i Exchange

sample[a]	solution	rate of ATP ⇌ P_i exchange ($s^{-1} \times 10^3$)
1	no additions	0.21
2	pyruvate kinase	0.24
3	2 mM ADP	0.21
2	5 mM ADP	0.18

[a] Reaction conditions for sample 1 were as for Figure 1 except that 2 mM phosphoenolpyruvate was present and the actin concentration was 2 μM. Sample 2 contained 50 μg/mL pyruvate kinase, sample 3 contained 2 mM MgADP, and sample 4 contained 5 mM MgADP. Ionic strength was kept constant by the addition of KCl.

concentration of medium ADP is shown in Table I. It can be seen that there is no significant change in the rate of ATP ⇌ P_i exchange with the addition of either 2 or 5 mM ADP or an ATP regenerating system (pyruvate kinase–phosphoenolpyruvate), which was found to keep the medium ADP level below 50 μM. White (1977) found the binding constant of ADP to acto-S1 to be 200 μM, and the effective binding constant will be very much weaker due to the competing effect of ATP which is present at a concentration more than 5000 times the K_m. The ADP concentration in the presence of a regenerating system is certainly not saturating, and the ADP incorporated into labeled ATP comes directly from hydrolysis of ATP without ADP dissociation. The pathway of reversal in which P_i binds first and ADP binds second does not occur to a measurable extent as this pathway also necessitates the binding of medium ADP. ATP ⇌ P_i exchange occurs by P_i binding to the state AM′·ADP and this is likely the dominant pathway in the forward direction but the present data do not allow the extent of ordering to be determined.

The dependence of the rate of ATP ⇌ P_i exchange on actin concentration is treated quantitatively under Experimental Procedure, but the results can be explained qualitatively in the following manner. The rate of exchange is $k_{-1}K_2[A] \times [M^*\cdot ATP^\dagger]$ (Sleep & Hutton, 1978). (The dagger symbol signifies that the γ-phosphate came from the medium.) Provided $k_{-1}K_2[A]$ is much less than the sums of the forward and reverse, first- or pseudo-first-order rates of the steps between M*·ATP and AM′·ADP, the relative concentrations of the two species will be governed simply by the equilibrium constants of the intervening steps. Actin is released to reach M*·ATP from AM′·ADP, and thus $[M^*\cdot ATP^\dagger]/[AM'\cdot ADP]$

$\propto 1/[A]$. The rate of release of ATP from M*·ATP $\propto [A]$, and these two sources of actin dependence cancel out. The rate of ATP $\rightleftharpoons$ P_i exchange is dependent on actin concentration via its effect on the steady-state concentration of AM′·ADP. As has already been discussed, AM′·ADP exists due to it being a steady-state intermediate and not from ADP rebinding, and thus release of ADP can be treated as an irreversible step. The fraction of S1 in the form AM′·ADP is $v/(v + k_6)$ where $v = V_{max}[A]/(K_m + [A])$ and k_6 is the rate of release of ADP from AM′·ADP. On this basis the rate of ATP $\rightleftharpoons$ P_i exchange will have the same actin dependence as the steady-state ATPase rate. This is a reasonably good description of the observed behavior (Figure 2), but some inhibition is observed at high actin concentration. This discrepancy arises from the equilibrium assumption made in this qualitative treatment; at a high enough concentration of actin, the M*·ATP† concentration will be less than the amount in equilibrium with AM′·ADP because the rate of release of ATP to the medium ($k_{-1}K_2[A]$) is not negligible compared to the rate of loss to M**·ADP·P_i (k_3); this accounts for the term $K_z + [A]$ in eq 6 which is approximately $K_3k_5K_4/(k_{-1}K_2) \gg 1$ and is proportional to $1 + k_{-1}K_2[A]/k_3$.

The upper line of Figure 2 is a theoretical curve [$V_{ex} = J[A]/[(K_m + [A])(K_z + [A])]$ where $J = k_{-3}[P_i]/(K_4K_5)(V_m/k_6)$ is the constant of proportionality in eq 6]. The value of K_m is taken from the steady-state ATPase measurements shown in Figure 2, and the value of K_z is taken from other acto-S1 experiments as detailed under Experimental Procedure. The quality of the fit is good considering that the only parameter used to fit the curve was J. The equations were derived for a simple Lymn–Taylor scheme without a refractory state (Chock et al., 1976), but a refractory-state model would give, at least to reasonable approximation, the same actin dependence because the rate of exchange would still be dependent on the same three factors, the steady-state concentration of AM′·ADP, the equilibrium constant between AM′·ADP and M*·ATP, and the rate of ATP release $k_{-1}K_2[A]$. The introduction of a refractory state will tend to lead to somewhat greater inhibition as the equilibrium assumption between M*·ATP† and AM′·ADP will break down earlier. It is because of the lack of discriminatory power of the data that the derivation is given for the simpler model.

Release of ATP from S1 by Actin. The actin dependence of ATP $\rightleftharpoons$ P_i exchange is that predicted on the basis of a simple Lymn–Taylor scheme. This is a useful point to establish for interpretation of exchange measurements of muscle fibers but in itself does not increase our understanding of the acto-S1 mechanism, as the rate of exchange is dependent on too many unknowns. The number of unknowns can be reduced by use of an independent measurement of the rate of release of ATP from S1 by actin. This rate has been determined for the S1-bound ATP state, M*·ATP, formed from ATP (Sleep & Hutton, 1978), but before this number is used to help interpret ATP $\rightleftharpoons$ P_i exchange data, it should be established that the bound ATP state formed from ADP and P_i is the same as the steady-state intermediate formed from ATP. The most favorable conditions for the $^{32}P_i$ experiment involve the use of a high concentration of S1 and a small reaction volume to allow adequate dilution into the solution of actin and unlabeled ATP. This precludes the use of the quench flow apparatus, and it seemed desirable to do the $^{32}P_i$ and [γ-^{32}P]ATP experiments under the same conditions for direct comparison.

At a moderate concentration of S1 the rate of binding of a substoichiometric amount of ATP is much faster than product release, and there is a time (1–3 s) when essentially all the ATP is bound but little product release has occurred. A substoichiometric amount of [γ-^{32}P]ATP is added to S1, followed 3 s later by perchloric acid or a large excess of unlabeled ATP or a mixture of actin and unlabeled ATP. The perchloric acid quench gives the total nucleotide in the form of [γ-^{32}P]ATP; the unlabeled ATP chase gives the amount of free or loosely bound [γ-^{32}P]ATP; the addition of actin and unlabeled ATP gives the fraction of tightly bound [γ-^{32}P]ATP released by actin. Actin releases a fraction of the bound [γ-^{32}P]ATP and the released [γ-^{32}P]ATP is subsequently lost as the pool is hydrolyzed. Extrapolation of the data points back to the time of actin addition allows the amount of ATP released to be determined. The solid lines of Figures 3 and 4 show that 36 and 41% of the tightly bound ATP were released by 3 and 10 μM actin, respectively.

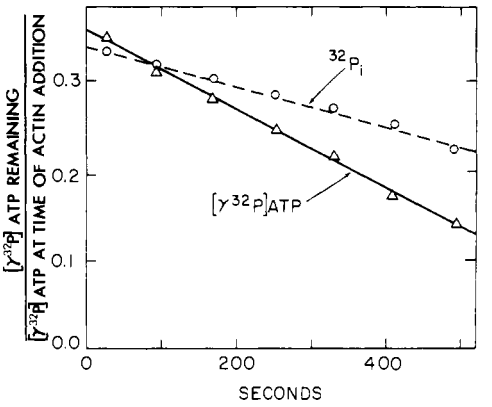


FIGURE 3: Release of bound ATP with 3 μM actin. Curve O shows the release of ATP from S1-bound ATP formed from ADP and $^{32}P_i$. 25 μL of $^{32}P_i$ (1 mM) was added to 50 μL of S1 (75 μM) and ADP (150 μM), and 2 mL of actin (3.45 μM) and MgATP (2.3 mM) was added after waiting 1 min to allow an equilibrium amount of S1-bound ATP to form. Concentrations refer to solutions prior to mixing. Samples (250 μL) were analyzed for the [^{32}P]ATP/$^{32}P_i$ ratio. At the time of the addition of actin, 0.012 of the S1 had ATP bound. In this experiment a proportion of the bound labeled ATP gets released to the medium, and the time course shows the hydrolysis of the medium ATP pool which has been labeled. A low P_i concentration was used in this experiment to minimize the difference in ionic strengths between the $^{32}P_i$ and [^{32}P]ATP experiments. Curve Δ shows the release of ATP from S1-bound ATP formed from [^{32}P]ATP. 50 μL of [^{32}P]ATP (12 μM) was added to 100 μL of S1 (19 μM), followed 3 s later by 1 mL of actin (3.45 μM) and MgATP (2.3 mM). An unlabeled ATP chase at 3 s showed that 1% of the nucleotide was free or loosely bound. A perchloric acid quench at 3 s showed that 21% of the nucleotide was in the form of ATP.

Addition of actin and unlabeled ATP to the S1-bound ATP state formed from ADP and P_i results in a fraction of the bound ATP being released. Comparison of the solid and dashed lines in Figures 3 and 4 shows that the percentage of the bound ATP released when it is formed from ADP and P_i is similar to, although slightly lower than, that released when formed from ATP, indicating that the bound ATP state formed by the two methods is the same. As is discussed below, an increase in counts from ATP $\rightleftharpoons$ P_i exchange in the $^{32}P_i$ experiments accounts for most of the difference between the two lines.

The counts in ATP extrapolated to the time of actin addition have been assigned to the release of bound ATP, but to make this assignment it is necessary to show that there is no burst of ATP formation associated with ATP $\rightleftharpoons$ P_i exchange as there is for S1 in the absence of actin (Wolcott & Boyer, 1974). The control is to reorder the addition of the constituents of the reaction mixture. In the ATP release experiment $^{32}P_i$ was added to a solution of S1, ADP, and P_i, and, when equilibrium had been established, an aliquot was added to actin and ATP.

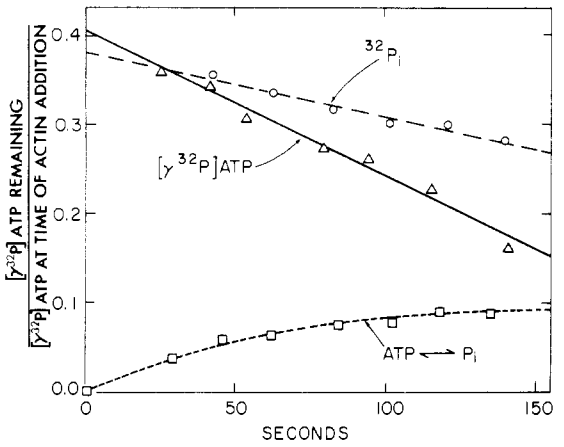


FIGURE 4: Release of bound ATP and ATP $\rightleftharpoons$ P_i exchange with 10 μM actin. The dashed (○) and solid (△) lines correspond to the experiments of Figure 3 except that 11.5 μM actin was added. The dotted line (□) is the ATP $\rightleftharpoons$ P_i exchange control. 50 μL of S1 (75 μM), ADP (150 μM), and P_i (1 mM) was added to 2 mL of actin (11.5 μM) and MgATP (2.3 mM), and 10 s later 25 μL of $^{32}P_i$ (1 mM) was added and samples were taken as for the $^{32}P_i$ experiment of Figure 1. The ordinate for this plot is on the same scale as the ATP release experiments: that is, ATP released to the medium via ATP $\rightleftharpoons$ P_i exchange is expressed in units of bound ATP existing at the time of actin addition when the reactants are added in the inverse order.

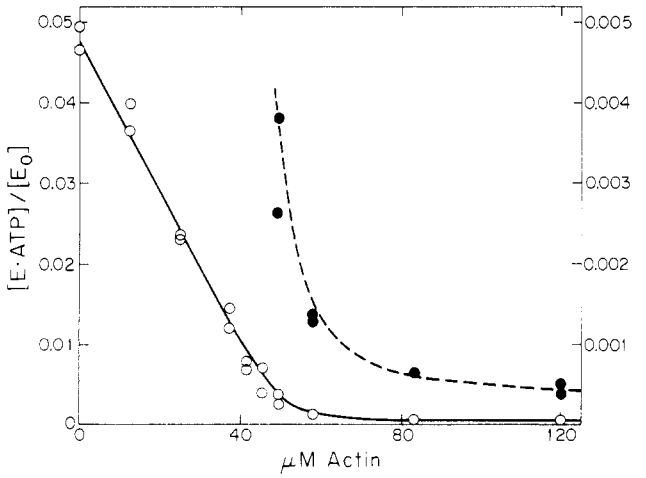


FIGURE 5: Inhibition of S1-bound ATP formation by actin. The fraction of S1 with ATP bound is plotted against the total actin concentration. The line is a theoretical curve based on the following values of equilibrium constants (eq 6): $K_a = 2 \times 10^4$ M^{-1}; $K_b = 0.1$ M; $K_c = 4 \times 10^6$ M^{-1}; $K_d = 20$ M. The dashed curve (●) is a replot in which the ordinate has been magnified by a factor of 10. Reaction conditions: 50 μM S1, 2 mM ADP, 3 mM $MgCl_2$, 5 mM P_i, 10 mM KCl, and 10 mM Hepes, neutralized to pH 7 with Tris.

The control was to add the S1, ADP, and P_i to the actin and ATP, 10 s prior to the addition of $^{32}P_i$. The dotted curve in Figure 4 shows the result of the ATP $\rightleftharpoons$ P_i exchange control experiment at 10 μM actin. The most important point is the lack of detectable burst of [γ-^{32}P]ATP formation, in agreement with the results of Wolcott & Boyer (1974) and Paulsen (1976). The initial level of labeled ATP in the main experiment is thus entirely due to release of ATP by actin from the equilibrium mixture of M*·ATP and M**·ADP·P_i. This control does not prove in general that a transient phase of enzyme-bound ATP synthesis or hydrolysis does not take place on actin addition to M*·ATP and M**·ADP·P_i. If $K_2 \neq K_4$ and the added actin is $\gtrsim 1/K_2$ or $\gtrsim 1/K_4$, the equilibrium between bound ATP and bound products would change. This would not be evident from our experiments which are only concerned with monitoring the steady-state hydrolysis of the ATP pool after it has been labeled.

The rate of ATP $\rightleftharpoons$ P_i exchange was 2×10^{-5} s^{-1} (0.036 mM P_i; 10 μM actin), in good agreement with the rate 1.4×10^{-3} s^{-1} [2 mM P_i; 10 μM actin (Figure 2)] when allowance is made for the different P_i concentrations by expression as a second-order rate constant $[(2 \times 10^{-5}\ s^{-1})/(36 \times 10^{-6}\ M) = 0.5\ M^{-1}\ s^{-1}$ (20 °C); cf. $(1.4 \times 10^{-3}\ s^{-1})/(2 \times 10^{-3}\ M) = 0.7\ M^{-1}\ s^{-1}$ (25 °C)]. The final S1 concentration was the same in the two experiments (using [γ-^{32}P]ATP or $^{32}P_i$), and thus in the absence of ATP $\rightleftharpoons$ P_i exchange the ATP counts would have extrapolated to zero at the same time, that of complete hydrolysis of the ATP pool.

Formation of S1-Bound ATP in the Presence of Actin. Actin binds more tightly to M*·ADP than to M*·ATP as shown by the fact that ADP has a very limited ability to dissociate acto-S1. For thermodynamic consistency, actin must inhibit the formation of enzyme-bound ATP from ADP and P_i. The experiment is technically difficult due to the high ratio of counts in P_i to counts in enzyme-bound ATP, and this necessitates using a high concentration of S1. A plot of ATP formed against actin concentration is given in Figure 5. The line is a theoretical curve based on the following values of equilibrium constants: $K_a = 2 \times 10^4$ M^{-1}; $K_b = 0.1$ M; $K_c = 4 \times 10^6$ M^{-1}; $K_d = 20$ M (eq 9).

$$\begin{array}{ccccc} M^*\cdot ATP & \overset{b\ P_i}{\rightleftharpoons} & M^*\cdot ADP & \overset{e}{\rightleftharpoons} & M + ADP \\ \downarrow a & & \downarrow c & & \downarrow f \\ AM\cdot ATP & \overset{d\ P_i}{\rightleftharpoons} & AM\cdot ADP & \overset{g}{\rightleftharpoons} & AM + ADP \end{array} \qquad (9)$$

The value of K_b is defined by the amount of ATP formed in the absence of actin, and the value obtained in this experiment (0.1 M) is in good agreement with a previous measurement under similar conditions [0.12 M (Sleep et al., 1978)]. The value of K_c is defined by the extent the curve deviates from a simple intersection of two straight lines at the point of 1:1 stoichiometry. This measurement is not of high accuracy, but the value obtained (4×10^6 M^{-1}) is quite reproducible and is as close as can be expected to the value determined from measurements of K_e, K_f, and K_g [$K_e = 10^{-6}$ M (Bagshaw & Trentham, 1974), $K_f = 10^8$ M^{-1} (Marston & Weber, 1975), and $K_g = 10^{-4}$ M (White, 1977), which together give a value of 10^6 M^{-1} for K_c; there is some variation in reaction conditions from parameter to parameter]. Previous measurements of K_c (Highsmith, 1976; L. Greene and E. Eisenberg, personal communication) have been made at much higher ionic strength and are correspondingly lower [$(1\text{–}3) \times 10^5$ M^{-1}; $I \approx 0.2$]. Our value for K_d (20 M) is defined by the amount of ATP remaining at high actin concentration. This estimate is best regarded as a useful upper limit rather than a strict estimate because some bound ATP could remain at high actin concentrations due to heterogeneity. The resultant value for K_a (2×10^4 M^{-1}) is in the range suggested by less direct but technically simpler experiments [$K_a \approx 4(1/K_m$ for actin)] (Stein et al., 1979).

Knowledge of the concentration of S1-bound ATP allowed a prediction of the rate of S1-catalyzed medium $P_i \rightleftharpoons$ HOH exchange which was in good agreement with the experimentally observed values (Webb et al., 1978; Sleep et al., 1978). The rates of acto-S1-catalyzed medium $P_i \rightleftharpoons$ HOH exchange found in preliminary experiments were more than 10 times slower than those predicted. This observation is consistent with those of Dr. D. R. Trentham (personal communication), who also found the rate of acto-S1 medium exchange to be extremely slow. Any exchange that did occur was not characterized by the O/P ratio expected from intermediate exchange

studies. At high actin concentrations after very long times, slight exchange characteristic of an O/P ratio of 1.0 was observed but it was found to be almost as great with actin in the absence of S1, suggesting that actin may catalyze medium $P_i \rightleftharpoons$ HOH exchange. Any such exchange would be expected to have an O/P ratio of 1.0 for we found that no intermediate exchange occurred (O/P = 1.0) on actin polymerization.

Discussion

The ATP $\rightleftharpoons$ P_i exchange of the simplest system, acto-S1, will be discussed first, followed by a consideration of the previously reported results of exchange experiments on the more complex systems such as actomyosin and muscle fibers.

The dependence of the rate of ATP $\rightleftharpoons$ P_i exchange on actin concentration was developed in terms of exchange starting by P_i binding to a state AM′·ADP, the concentration of which is linearly dependent on the steady-state ATPase rate. If P_i binds to AM·ADP, the state formed from medium ADP, then the dependence of the rate of exchange on medium ADP concentration and also the absolute rate of exchange can be predicted. Poor agreement between the experimentally observed and predicted values would indicate that AM′·ADP is not the same as AM·ADP but is a second state which cannot be formed in significant concentrations from medium ADP due to the equilibrium constant between AM′·ADP and AM·ADP favoring AM·ADP.

Dependence of the Rate of ATP $\rightleftharpoons$ P_i Exchange on Medium ADP Concentration. At 2 μM actin the steady-state ATPase rate is 3.7 s^{-1} (Figure 2). The rate of release of ADP from the AM·ADP state which can be formed from medium ADP is 500 s^{-1} at 4 °C but unmeasurably fast (>1000 s^{-1}) at 20 °C (White, 1977). For these calculations a rate of 2000 s^{-1} (25 °C) will be used, which means that $3.7/2000 \simeq 1/500$ of the enzyme is in the form AM·ADP (see eq 5) due to it being a steady-state intermediate. The K_m for ATP predicted from transient measurements is 0.6 μM [3.7 $s^{-1}/(6 \times 10^6$ M^{-1} $s^{-1})$; the value of the apparent second-order rate constant of ATP binding is an extrapolation of the data of White & Taylor (1976)]. The ATP concentration used for the exchange experiment was 2 mM (Figure 2) which is 3000 times the K_m. The ADP dissociation constant is 200 μM (White, 1977), and the highest ADP concentration used was 5 mM which is 25 times the dissociation constant. The fraction of the enzyme which is in the form AM·ADP due to medium ADP binding is thus $25/3000 \simeq 1/100$. A 1% reduction in the ATPase rate due to ADP competition would not be detectable, but the rate of ATP $\simeq$ P_i exchange is linearly dependent on the concentration of AM′·ADP. If the rate of exchange were equal to v'[AM·ADP], then the addition of 5 mM ADP would increase the rate by a factor of 6 [from $v'(1/500)$ to $v'(1/500 + 1/100)$]. No such increase is observed (Table I) and the only resonable explanation is that AM′·ADP is not the same as AM·ADP. If the equilibrium constant between the AM·ADP states were 5 in favor of AM·ADP, then the exchange rate would be predicted to double [$v'(1/500 + 1/500)$] in the presence of 5 mM ADP. The value of 5 thus serves as a safe lower limit for this equilibrium constant and the true value is probably more than 20.

A second AM·ADP state can account for the lack of effect of medium ADP on the rate of ATP $\rightleftharpoons$ P_i exchange. Is this the only explanation or are there sufficient variables in the refractory state nondissociating model of Stein et al. (1979) to explain the results without the introduction of an extra AM·ADP state? The lack of effect of medium ADP proves that the concentration of AM′·ADP is unaffected by medium ADP. The concentration of the state formed from medium ADP is predicted to increase by a factor of 6, and thus either the state has been incorrectly characterized, which is unlikely, or P_i binds to a higher energy AM·ADP state. Complexities in the earlier part of the scheme have no effect on this conclusion.

Prediction of the Absolute Rate of ATP $\rightleftharpoons$ P_i Exchange. Is the absolute rate of exchange that expected if exchange started from AM·ADP, the state formed from medium ADP? The rate of exchange is $k_{-1}K_2$[A][M*·ATP†]. At 2 μM actin [AM·ADP]/M_0 = 1/500 as shown earlier (M_0 is the total concentration of S1). At low actin concentrations where $k_{-1}K_2$[A] $\ll k_3$, the concentration of M*·ATP† is well approximated by the concentration in equilibrium with AM′·ADP. If this state were the same as AM·ADP, then

$$\frac{[M^*\cdot ATP^\dagger]}{[AM\cdot ADP]} = \frac{[P_i]}{K_b K_c [A]}$$

$$= (2.5 \times 10^{-6})[P_i]/[A] \text{ (see eq 9)}$$

$$= 2.5 \times 10^{-3} \text{ (at 2 mM } P_i \text{ and 2 } \mu\text{M actin)}$$

$$\frac{[M^*\cdot ATP^\dagger]}{M_0} = \frac{[M^*\cdot ATP^\dagger]}{[AM\cdot ADP]} \frac{[AM\cdot ADP]}{M_0} =$$

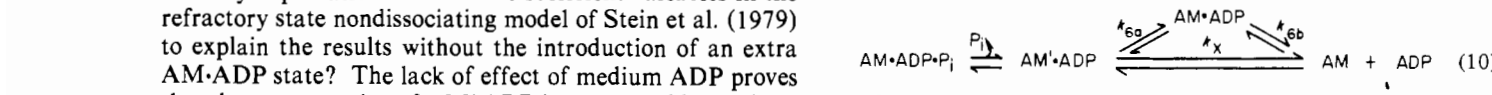
$$(2.5 \times 10^{-3})(1/500) = 5 \times 10^{-6}$$

The value of $k_{-1}K_2$[A] is $(9 \times 10^5$ M^{-1} $s^{-1})(2 \times 10^{-6}$ M) = 1.8 s^{-1}; thus the predicted rate of exchange is $1.8(5 \times 10^{-6}) = 9 \times 10^{-6}$ s^{-1}. This rate is a factor of 50 times slower than the observed rate of 4.8×10^{-4} s^{-1}, again providing evidence for the existence of a second AM·ADP state.

Can the data be explained with other models? Consider the situation at a low actin concentration. The rate of ATP $\rightleftharpoons$ P_i exchange is k_{-1}[AM·ATP†] or, equivalently, $k_{-1}K_2$[A][M*·ATP†]. The concentration of M*·ATP† cannot be greater than that in equilibrium with AM′·ADP. The nature of intervening pathways can reduce the concentration of M*·ATP† to below the equilibrium value but cannot increase it. The discrepancy between predicted and observed rates of ATP $\rightleftharpoons$ P_i exchange cannot be eliminated by the introduction of a refractory state or a nondissociating pathway.

The acto-S1 ATP $\rightleftharpoons$ P_i exchange experiments of Cardon & Boyer (1978) provide further evidence that AM′·ADP is not the same as AM·ADP. They worked at higher ionic strength (I = 0.21) which weakens the interaction between actin and S1 states and thus reduces the inhibition of bound ATP formation. It also reduces the ATPase rate and with it the AM·ADP concentration. The slow ATPase rate, however, facilitates the use of high S1 concentrations and allowed measurement of a burst of ATP formation which was >100 times larger than would be predicted if P_i bound to AM·ADP but can be explained in terms of P_i binding to a second state AM′·ADP.

The ATP $\rightleftharpoons$ P_i exchange experiments provide only a very limited characterization of the state AM′·ADP. A factor of 50 in the exchange rate can be gained either by the steady-state concentration of AM′·ADP being 50 times that of AM·ADP or because the equilibrium constant between the states AM′·ADP and AM·ADP is 50 in favor of the latter or by a combination of the two. As the state AM·ADP has not been proved to be on the pathway, the states are drawn in the form of a triangle (eq 10) to show the possibility that the formation



(10)

of AM·ADP is a side reaction. Satisfying the formula $K_{6a}k_{6b}/(k_{6a} + k_x)$ would give the observed 50-fold enhance-

ment of rate. The lack of effect of medium ADP on the rate of ATP $\rightleftharpoons$ P_i exchange means that at least part of the effect must arise from AM′·ADP being of higher free energy (i.e., $K_{6a} > 5$, as discussed earlier). A second reason why K_{6a} must be significantly greater than 1 is that if $K_{6a} = 1$, then White (1977) would have observed two classes of ADP dissociating from AM, one at a rate 50 times slower than the other. Bagshaw et al. (1974) characterized two myosin·ADP states (M*·ADP and M·ADP), but the state AM·ADP would have to correspond to M*·ADP rather than M·ADP which would leave no equivalent for AM·ADP. However, a more detailed study of the kinetics of fluorescence enhancement (Trybus & Taylor, 1979) is consistent with their being three myosin·ADP states, and two of these may correspond to AM′·ADP and AM·ADP.

The existence of the extra state does appear to be of interest in connection with the Huxley–Simmons model of contraction in which there is more than one step involving a change in angle of the myosin head while bound to actin. It was known that a large fraction of the basic free energy change of ATP hydrolysis occurred in that part of the mechanism between AM·ADP·P_i and AM·ADP (White, 1977), but while there was evidence for only a single biochemical step, two or more mechanical steps had been postulated (Huxley & Simmons, 1971).

Release of ATP from S1 by Actin. The results reported here show that the ATP state formed from ADP and P_i is the same as that formed from ATP. This result was expected but the confirmation is not trivial as it justifies the assumption made in measuring the binding constant of ATP to S1.

The result is also of interest in emphasizing the similarity of the mechanochemical actomyosin system and ion pumps which use chemical energy to perform osmotic work. The first experiments were done on the Na^+/K^+ pump by Taniguchi & Post (1975), but similar experiments were done on the sarcoplasmic reticulum (SR) Ca^{2+} pump by Knowles & Racker (1975), who refined certain features of the experiment. The latter paper shows that the addition of Ca^{2+} and ADP to the phosphorylated intermediate E–P, formed by the addition of P_i to SR in the absence of Ca^{2+}, resulted in the release of ATP to the medium. This is analogous to adding actin to M*·ATP, which is formed by the addition of ADP and P_i to myosin in the absence of actin. The analogous behavior of Ca^{2+} and actin and the states E–P and M*·ATP shows that there are fundamental similarities in the energetics of the two systems.

Comparison of Actomyosin and Acto-S1 ATP $\rightleftharpoons$ P_i Exchange. The difference in the kinetics of acto-S1 and actomyosin ATPase in the forward direction is very marked (Burke et al., 1974), and it is of interest to compare the rates in the reverse direction. Wolcott & Boyer (1974) found a ratio of rates of ATP hydrolysis to ATP $\rightleftharpoons$ P_i exchange of 500 at 60 mM KCl and 50 mM P_i, and Paulsen (1976) found a similar ratio at a much lower ionic strength and 2 mM P_i. The agreement between the two ratios is fortuitous and arises from the opposing effects of P_i concentration and ionic strength.

Our exchange measurements on acto-S1 were done at approximately the same ionic strength and P_i concentration as the experiments of Paulsen. The ratio of forward to reverse rates was constant at low actin concentrations and was 4000, a factor of 8 larger than for actomyosin. At the highest actin concentration used, the ratio was 20000, a factor of 40 greater than for actomyosin. It is not possible to make a definitive comparison of the acto-S1 and actomyosin rates due to the differences in K_m for actin and ionic strength dependencies; the results do, however, suggest that the rate of ATP $\rightleftharpoons$ P_i exchange of actomyosin is significantly greater than that of acto-S1.

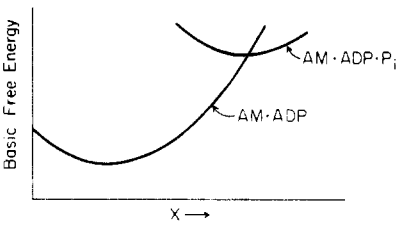


FIGURE 6: Basic free energy profile for the Eisenberg–Hill model of cross bridge action. x is the relative position of the filaments.

ATP $\rightleftharpoons$ P_i Exchange in the Organized System. Gillis & Marechal (1974) and Ulbrich & Ruegg (1977) have investigated the incorporation of $^{32}P_i$ into ATP by muscle fibers to determine whether work done on a fiber can be converted into chemical energy (Hill & Howarth, 1959). Significant incorporation was observed, and the question of whether this was a "chemomechanical energy transformation" or simply ATP $\rightleftharpoons$ P_i exchange was considered. As shown in this paper, even the completely uncoupled acto-S1 system catalyzes ATP $\rightleftharpoons$ P_i exchange, and coupling of the ATPase to mechanical work would be expected to result in the enhancement of this rate. It seems best to reserve the term "chemomechanical energy transformation" for cases where the net flux is in the direction of synthesis.

The rate of ATP $\rightleftharpoons$ P_i exchange can be considered in terms of dependence on three factors: first, the concentration of AM·ADP, the species to which P_i binds; second, the basic free energy change between AM·ADP and M*·ATP; third, the rate of release of ATP from M*·ATP. To consider the effect of going from the soluble acto-S1 system to a muscle fiber, it is convenient to use the most biochemical of muscle models (Eisenberg & Hill, 1978). Force is produced by a state AM‡D undergoing a transition to AMD without a change in the relative position of the filaments, x. The orientation of the myosin head no longer corresponds to an energy minimum and force is exerted (Figure 6). White (1977) determined the binding constant of ADP to acto-S1 and established that the basic free energy change between AM·ADP·P_i and AM·ADP was about half the total basic free energy change of ATP hydrolysis. If AM·ADP·P_i $\rightarrow$ AM·ADP is a step in the cycle, i.e., if P_i is released prior to ADP, then Eisenberg and Hill's states AM‡D and AMD are most plausibly taken as AM·ADP·P_i and AM·ADP. The ATP $\rightleftharpoons$ P_i exchange measurements reported in this paper suggest an ordered mechanism, thus favoring this assignment.

The rate of ATP $\rightleftharpoons$ P_i exchange of an isometric muscle would be expected to be higher than that of acto-S1 for two reasons. First, it is the state AM·ADP which exerts the force, and thus one would expect its concentration to be enhanced during an isometric contraction. Second, the average energy of the state AM·ADP is higher for a fiber producing tension than for acto-S1 as the orientation of the head in the fiber cannot correspond to an energy minimum, whereas this would be the orientation expected for acto-S1. These predictions are borne out for although Gillis & Marechal (1974) did not directly measure the ATPase rate they estimated that the ratio of ATPase to ATP $\rightleftharpoons$ P_i exchange rates was in the range 100–1000 ($[P_i]$ = 1.67 mM), and Ulbrich & Ruegg (1976) observed a ratio of 100 for insect muscle fiber ($[P_i]$ = 3–4 mM). These ratios are to be compared with the ratio of $\geq$4000 we observed for acto-S1 ($[P_i]$ = 2 mM). Gillis & Marechal (1974) observed that the rate of exchange increased with

tension, and this is what would be expected because the average energy and/or the number of cross bridges in the force-producing state, AM·ADP, must have increased. It was observed earlier that actomyosin catalyzes a higher rate of ATP $\rightleftharpoons$ P_i exchange than acto-S1. The intermediate oxygen exchange results of Sleep et al. (1980) can only be explained if myosin has a high local concentration of actin surrounding it. Under these conditions filaments must interact at several points and experience mechanical constraints. It seems likely that actomyosin resembles a muscle to some extent even prior to superprecipitation, and this would account for the enhanced rate of ATP $\rightleftharpoons$ P_i exchange.

In summary, the rate of ATP $\rightleftharpoons$ P_i exchange of a muscle fiber is dependent on features of considerable interest, such as the basic free energy change of a restricted segment of the ATPase mechanism, but due to the dependence on several such features, all of which are at present unknown, it is only possible to consider such results in qualitative terms.

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