Single Turnovers of Adenosine 5′-Triphosphate by Myofibrils and Actomyosin Subfragment 1†

J. A. Sleep

ABSTRACT: The ATPase of myofibrils has been investigated by using single-turnover methods which avoid contraction to unphysiologically short sarcomere lengths. A substoichiometric amount of ATP is mixed with myofibrils in rigor, and the rate of decay of bound ATP to ADP and P_i is followed. At 0 °C, the rate is 0.45 s^{-1} and is not dependent on ionic strength in the range I = 0.035–0.175. The steady-state ATPase of myofibrils under these conditions is considerably slower (0.072 s^{-1}) than the rate of ATP decay and must be controlled by a subsequent step. Unlabeled ATP chase experiments were used to determine the rate of release to the medium of bound ATP. This rate is comparable to that of hydrolysis and dissociation as products but is dependent on ionic strength, changing from 0.53 s^{-1} at I = 0.035 to 0.12 s^{-1} at I = 0.085. Knowledge of the rate of release of bound ATP, together with the rate of ATP binding, allows the basic equilibrium constant between the states AM + ATP and A + M*·ATP to be estimated. The value is 3×10^4, which corresponds to about 40% of the total basic free-energy change between physiological concentrations of medium ATP and medium ADP and P_i. Single turnovers of acto-S1 ATPase were also investigated. At 0 °C and I = 0.02, the rate of decay of bound ATP was equal to the rate of steady-state ATPase over a range of actin concentrations up to 10 times the K_m. This observation suggests that it is the same enzymatic step which controls the rates of both processes; acto-S1 models of this type are discussed.

Lymn & Taylor (1971) proposed a biochemical model for actomyosin ATPase in which actin and myosin associate and dissociate once per ATP molecule hydrolyzed: the model was thus readily consistent with the proposed mechanical cross-bridge cycle (Huxley, 1969). Since then, the main points of the model have been shown to be valid at least at low actin concentration (White & Taylor, 1976; Sleep & Taylor, 1976). Eisenberg and collaborators have investigated the kinetics at saturating concentrations of actin and have suggested the existence of a refractory state which serves to keep the system dissociated under these conditions (Eisenberg et al., 1972; Chock et al., 1976). More recently, they have obtained data consistent with the occurrence of a pathway of ATP hydrolysis in which actin does not dissociate from myosin (Stein et al., 1979). Progress in understanding the kinetics and mechanism of acto-S1 ATPase has thus been steady, but in measuring the rates of elementary steps of actomyosin ATPase in muscle fibers, where the reaction is coupled to the performance of mechanical work, it has been slow.

Myofibrils are a potentially useful intermediate between soluble acto-S1 and organized muscle fibers; they retain the filament structure of muscle but are more susceptible to the methods of solution chemistry. In this paper, experiments on single turnovers of ATP by myofibrils are reported. Myofibrils are mixed with ATP in rigor, the concentration of myosin heads being in excess of that of ATP: the excess of heads remains in rigor and prevents sliding of the filaments, so approximating an isometric contraction. Single-turnover methods thus avoid a common difficulty of myofibril experiments; if there is an excess of ATP, myofibrils contract to a much shorter sarcomere length than occurs naturally, with a corresponding irreversible loss of structure.

To understand the information that can be gained from myofibril single-turnover experiments, we will first consider the application of the single-turnover technique to S1 (Bagshaw & Trentham, 1973). The rate-limiting step occurs after product formation (k_b = 0.05 s^{-1} at 20 °C, eq 1), which accounts for the early P_i burst (Lymn & Taylor, 1970). The apparent second-order rate constant of ATP binding to S1 (k_a) is 2×10^6 M^{-1} s^{-1}, and thus if a substoichiometric amount of ATP is added to S1 (e.g., 3 μM ATP to 10 μM S1), the rate of ATP binding [$>(10 - 3) \times 10^{-6}$ M $\times$ 2×10^6 M^{-1} s^{-1} = 15 s^{-1}] is much faster than k_b. Bagshaw and Trentham observed that if the reaction was quenched in acid at a time (t = 1–2 s) when all the ATP had bound, but little product release had occurred (i.e., $t \ll 1/k_b$), 10% of the nucleotide was in the form of ATP. Acid quenching at later times showed that the bound ATP decayed at the steady-state ATPase rate. The bound ATP can be accounted for if the equilibrium constant for ATP hydrolysis on the enzyme (K_3) is 9 [(100 − 10)/10]. Equilibrium between M*·ATP and M**·ADP·P_i is preserved because $k_3 + k_{-3} \gg k_b$, and thus bound ATP decays at the steady-state rate $k_b K_3/(1 + K_3)$. (More generally, bound ATP decays at the net forward rate from M**·ADP·P_i times the fraction of enzyme in the form M**·ADP·Pi.) If instead of acid quenching, the reaction was chased at 2 s with an excess of unlabeled ATP and 2 min [$>5(1/k_b)$] was allowed for completion of the turnover, then all the ATP was found to be hydrolyzed. The latter experiment confirmed that the ATP observed was enzyme bound and further that the bound ATP was committed to hydrolysis. Release of bound nucleotide is controlled by the ratio k_{-a}:$K_3 k_b$, and as no ATP is released, $k_{-a} \ll K_3 k_b$. An analogous experiment to the unlabeled ATP chase was reported by Sleep & Hutton (1978). In the presence of actin, bound ATP (M*·ATP) was found to be no longer committed to hydrolysis but to be exchangeable with medium ATP. In this case, unlabeled ATP chase experiments allowed determination of the rate of dissociation of bound ATP.

$$\mathrm{M} + \mathrm{ATP} \overset{a}{\rightleftharpoons} \mathrm{M^*{\cdot}ATP} \overset{3}{\rightleftharpoons} \mathrm{M^{**}{\cdot}ADP{\cdot}P_i} \overset{b}{\longrightarrow} \rightarrow \mathrm{M} + \text{products} \quad (1)$$

For myofibrils, the decay of bound ATP monitored by acid-quenched single turnovers should again give the net forward rate from the myosin product state. In the simple Lymn–Taylor scheme, this step is actin binding (M**·ADP·P_i + A → AM·ADP·P_i), and thus the experiment would measure

† From the Department of Physiology, University College London, London WC1E 6BT, England. *Received March 6, 1981.*

0006-2960/81/0420-5043$01.25/0

the net rate at which myosin heads bind to actin filaments in the cross-bridge cycle. With this interpretation, the effective actin concentration ($[A_{eff}]$) of a myofibril can be defined as that concentration which when multiplied by the solution value of the apparent second-order rate constant of actin binding to M**·ADP·P_i (k_4^a; White & Taylor, 1976) gives the observed first-order rate for myofibrils. It will be necessary, however, to consider other interpretations.

The unlabeled ATP chase experiments on myofibrils give the rate of dissociation of bound ATP, probably from the state M*·ATP via AM·ATP (AM + ATP $\overset{1}{\rightleftharpoons}$ AM·ATP $\overset{2}{\rightleftharpoons}$ A + M*·ATP). In this case, the rate is $k_{-1}K_2[A_{eff}]$, which is combination with the rate of ATP binding, $k_1[ATP]$, gives the basic equilibrium constant between AM + ATP and A + M*·ATP ($k_1[ATP]/k_{-1}K_2[A_{eff}]$). This indirect, kinetic estimate is useful because the value cannot be deduced from acto-S1 measurements when the effective actin concentration is unknown. The value of the free-energy change for this step turns out to be quite a large proportion of the total basic free-energy change of ATP hydrolysis under physiological conditions. This energy is unlikely to be converted to mechanical work unless the product of the ATP binding step (AM·ATP) produces more force than the reactant (AM).

An interesting feature of the results is that the rate of decay of bound ATP via hydrolysis (0.45 s^{-1} at 0 °C) is independent of ionic strength in the range 0.035–0.175, and this rate is about the same as the ATPase rate of acto-S1 at saturating actin concentration (V_m). These observations are not readily consistent with the simple Lymn–Taylor scheme, and it seemed of value to do the equivalent experiments for acto-S1.

For single turnovers with acto-S1, as the actin concentration is raised, what is the maximum rate of decay of bound ATP? A simple interpretation of the Lymn–Taylor scheme as originally proposed would predict that the maximum rate of decay equals the rate of the hydrolysis step k_3 because, on addition of saturating concentrations of actin ($[A] \gg 1/K_4$)

$$\text{AM} + \text{ATP} \overset{1}{\rightleftharpoons} \text{AM·ATP} \underset{2}{\overset{A}{\rightleftharpoons}} \text{M}^*\text{·ATP} \overset{3}{\rightleftharpoons}$$

$$\text{M}^{**}\text{·ADP·P}_i \underset{4}{\overset{A}{\rightleftharpoons}} \text{AM·ADP·P}_i \overset{5}{\rightleftharpoons} \text{AM} + \text{ADP} + \text{P}_i \quad (2)$$

M**·ADP·P_i would immediately become AM·ADP·P_i, allowing M*·ATP to decay to M**·ADP·P_i at k_3. The steady-state ATPase rate under these conditions is approximately k_5. In the original refractory state scheme (eq 3) as proposed by

$$\rightarrow \text{M}^*\text{·ATP} \overset{3a}{\rightleftharpoons} \text{M·products(R)} \overset{3b}{\rightleftharpoons} \text{M·products(NR)} \underset{4}{\overset{A}{\rightleftharpoons}}$$

$$\text{AM·products} \rightarrow \quad (3)$$

Eisenberg & Kielley (1973), the refractory state M·products(R) does not associate with actin. At saturating actin concentrations, the refractory to nonrefractory transition, step 3b, is the major rate-limiting step for the steady-state ATPase and also limits the rate of ATP cleavage during an acto-S1 single turnover.

Experimentally, the rate of ATP decay was found to be equal to the steady-state ATPase rate over a wide range of actin concentrations up to many times the K_m, and this observation is consistent either with the original refractory-state scheme or with schemes for which, at saturating concentrations of actin, ATP is hydrolyzed without the dissociation of myosin.

Materials and Methods

Materials. Myofibrils were prepared from the back muscle of rabbit, essentially as described by Perry & Grey (1956). The medium used to homogenize the muscle and for the first resuspension contained 5 mM EDTA to minimize shortening of the myofibrils. The concentration of myofibrils was determined by dissolving them in 5% sodium dodecyl sulfate and measuring OD_{280} ($E^{1\%}_{280nm}$ = 7.0; Sutoh & Harrington, 1977). Half the protein was taken to consist of myosin (Arata & Tonomura, 1976), for which a molecular weight of 460 000 was used. Myosin was prepared by the method of Perry (1955) from the back and hind leg muscles of rabbit. Myosin subfragment 1 was made by chymotryptic digestion (Weeds & Taylor, 1975). Actin was prepared by the method of Drabikowski & Gergely (1964). Protein concentrations were determined by the absorption at 280 nm, using the following extinction coefficients and molecular weights: S1, 0.77 cm^2/mg and 115 000; actin, 1.09 cm^2/mg and 42 000. [γ-^{32}P]ATP was made essentially by the method of Schendel & Wells (1973) or purchased from Amersham.

Methods. The myofibril single-turnover experiments were carried out with a delay line quench flow machine. An air cylinder forces the contents of two syringes containing myofibrils and a substoichiometric amount of [γ-^{32}P]ATP through a T mixer and into a delay line, where the reaction is allowed to continue. After a preset interval, a second air cylinder expels the contents of two more syringes containing buffer and the acid quench or chase solution. The buffer is used to force the contents of the delay line to mix with the quench or chase solution and to expel the mixture into a test tube. The syringes, mixing block and delay lines, were kept at a constant temperature. The samples so obtained were analyzed for the ratio of ^{32}P counts in ATP and P_i after separation with anion-exchange columns (Sleep & Hutton, 1978).

The sarcomere length of a typical myofibril preparation was 2.0–2.2 μm, and after a single-turnover experiment, in which the reaction was terminated with EDTA after 30 s, the sarcomere length was found to be unchanged.

The acto-S1 double-mixing experiments were performed in a stirred, temperature-controlled glass vial. To 100 μL of S1 (6 μM) was added 20 μL of [γ-^{32}P]ATP (9 μM); after 10 s, 225 μL of actin was added from a pneumatically driven automatic pipet, and the reaction was quenched with a pneumatically driven syringe at a series of later times. For the low actin concentrations, larger volumes of actin were added in order to dilute the S1 so that actin was in sufficient excess to make the reaction a reasonable approximation to first order. The time between the addition of actin and quenching was determined from a chart recorder. The stirrer was operated with a foot switch, and the vial was stirred vigorously for 3–5 s around the time of mixing. Tips for the automatic pipets were kept in a glass vial in ice, and the contents (actin or ATP) were exposed to room temperature for less than 10 s. The samples were analyzed as for the myofibril experiments. The steady-state rate of acto-S1 ATPase was determined by using [γ-^{32}P]ATP, separation of ATP and P_i being by charcoal adsorption.

Results

Acid-Quenched Single-Turnover Experiments. This experiment consists of the mixing of [γ-^{32}P]ATP and a suspension of myofibrils in rigor, followed by a perchloric acid quench. Typical concentrations after mixing were 5 μM ATP and 15 μM myosin heads. The fraction of label remaining as ATP is plotted against quench time in Figure 1. After 1 s, about 25% of the ATP remains, and most of this decays over the next 15 s. It is evident that there are two parts to the observed decay, a large component with a half-life of about 2 s and a small component with a half-life of about 40 s (see Figure 2). The effect of actin activation is clear because the

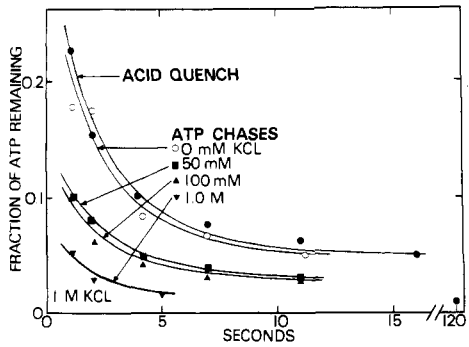


FIGURE 1: Single turnovers of myofibrillar ATPase. Myofibrils (0.25 mL, 15 mg/mL ≈ 33 μM myosin heads) were mixed with [γ-^{32}P]ATP (0.25 mL, 10 μM), and at the times indicated, the reaction was either acid quenched or chased with unlabeled ATP. Reaction conditions: 100 mM KCl, 20 mM imidazole, 3 mM $MgCl_2$, and 100 μM $CaCl_2$, pH 7, 0 °C. (●) Reaction mixture quenched with 2.2 mL of 0.5 M perchloric acid, 5 mM EDTA, 1 mM P_i, and 0.5 mM ATP. The fast phase of the curve is $y = 0.254 \exp(-0.45t)$. (▼) Reaction chased with 2.2 mL of 1 M KCl, 2 mM ATP, 3 mM $MgCl_2$, 50 mM Tris, and 100 μM $CaCl_2$, pH 8.5, 25 °C. The reaction was quenched with 1 mL of 2 M perchloric acid, 10 mM EDTA, and 2 mM P_i 10 s after the addition of the chase. (▲) Reaction chased with 100 mM KCl, 2 mM ATP, 3 mM $MgCl_2$, 20 mM imidazole, and 100 μM $CaCl_2$, pH 7, 0 °C, and stopped with 1 mL of 2 M perchloric acid 30 s later. Curve: $y = 0.104 \exp(-0.45t)$. (■) Same conditions as those for (▲), but chase contained 50 mM KCl. Curve: $y = 0.115 \exp(-0.45t)$. (○) Same conditions as those for (▲), but chase contained no KCl and 5 mM imidazole. Curve: $y = 0.236 \exp(-0.45t)$.

rate of the fast phase is about 20 times greater than that for S1 in vitro. This enhanced rate is approximately equal to the acto-S1 ATPase rate at saturating actin concentration (V_m). This observation, when considered in conjunction with the results of the acto-S1 single-turnover experiments described later, suggests that the effective actin concentration of a myofibril is greater than the K_m for actin of acto-S1 ($K_m \approx$ 1 mM at physiological ionic strength).

Before further treatment of the more interesting fast phase, the source of the slow phase will be considered. The slow phase of the decay is shown in Figure 2 and can be fitted by $y = 0.06 \exp(-0.0175t) + 0.02$. This rate (0.0175 s^{-1}) is essentially that expected for myosin in the absence of actin, and the simplest explanation of the slow phase is that part of the ATP binds to free myosin heads rather than actin-bound heads and consequently that actin is unable to accelerate product release. Consideration of the kinetics of ATP binding shows that it is the relative rate of binding and not the tightness of binding which controls the distribution of ATP among the two myosin species (free and actin bound). At 20 °C, the apparent second-order rates of ATP binding to S1 and to acto-S1 are similar (Lymn & Taylor, 1971), but at 0 °C, the latter rate is 20 times faster (Sleep & Taylor, 1976). This was an important reason why 0 °C was chosen for these experiments. The factor of 20 difference in rates in combination with the observation that most of the myosin heads are actin bound in a rigor muscle (Thomas et al., 1978; Cooke & Francks, 1980) suggests that ATP binding to free myosin will not be a serious problem but it would not be surprising if a small proportion of ATP bound to myosin. Preincubation with ADP or PP_i provides a test of the plausibility of this explanation. The ligand ADP binds tightly to S1 (5 μM) and is released slowly (0.02 s^{-1}; Bagshaw & Trentham, 1974) whereas it binds more weakly to acto-S1 (200 μM) and is released rapidly (400 s^{-1}; White, 1977). In these circumstances, 70 μM ADP should saturate any free myosin heads but only bind to a small proportion of actin-bound heads. The binding characteristics of PP_i [illegible] even more favorable, tighter to S1 (0.5 μM) and

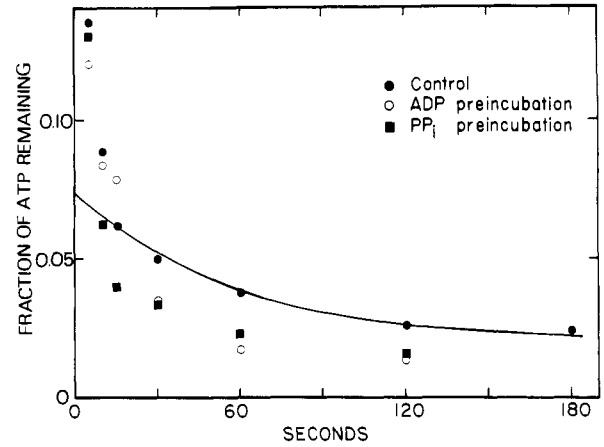


FIGURE 2: Effect of ADP and pyrophosphate preincubation. [γ-^{32}P]ATP (50 μL, 40 μM) was added to a small stirred tube of myofibrils (250 μL, 15 mg/mL) at the times indicated, and the solution was quenched with 1 mL of the 0.5 M perchloric acid quench solution. Reaction conditions: 50 mM KCl, 20 mM imidazole, 3 mM $MgCl_2$, and 100 μM $CaCl_2$. (●) Control. Curve: $y = 0.053 \exp(-0.0175t) + 0.02$. (○) ADP preincubation. ADP (12.5 μL) and diadenosine pentaphosphate (final concentrations 70 and 20 μM) were added to myofibrils 1 min prior to the addition of labeled ATP. (■) Pyrophosphate preincubation. Same conditions as for the ADP preincubation but 70 μM pyrophosphate and no diadenosine pentaphosphate.

weaker to acto-S1 (330 μM; Greene & Eisenberg, 1980). Figure 2 shows that preincubation has little effect. Preincubation might fail to demonstrate that the small-amplitude slow phase is due to free myosin for two reasons. First, the acto-S1 parameters may not be appropriate for fibers. Second, preincubation may channel ATP toward actin-bound heads, but some of these heads, having dissociated, may be unable to reassociate with their former actin monomers due to slight relative movement of the filaments so that product release occurs at the myosin and not the actomyosin rate. An alternative source of the slow phase is the presence of a contaminant ATPase. The most obvious possibilities are the Ca^{2+} pump of the sarcoplasmic reticulum and the H^+ pump of the mitochondria. They are made less likely by the observation that washing the myofibrils in 0.5% Triton does not affect the result. A further argument against the mitochondrial ATPase is the lack of effect of azide. The source of the slow phase remains uncertain, but its amplitude is sufficiently small compared to that of the fast phase that it does not seriously perturb analysis of the latter.

To interpret the large-amplitude fast phase of the decay, it is necessary to know that the ATP present during the decay phase is bound to myosin heads and not free in the medium. This was investigated by means of unlabeled ATP chase experiments, which are considered in detail below. However, the chase in 1 M KCl shows that at least 95% of the added ATP is bound after 1 s and that unbound ATP does not seriously affect the results. The slow phase must be subtracted to fit the fast phase. The difference in rate between the two phases is such that a small error in the rate of the slow process leads to a negligible error in the rate of the fast phase. The slow phase was not followed in all experiments, and the data of Figures 1 and 2 were obtained with different myofibril preparations. The fast phases of Figure 1 were fitted by subtracting a slow phase of the average observed rate (0.017 s^{-1}) and with an amplitude such that on subtraction the fast phase was reduced to zero in the time range 10–15 s (more than 5 times the half-life of the fast phase). The rate of the fast phase of the 100 mM KCl acid quench is 0.45 s^{-1}. Experiments at higher temperatures proved more difficult due to significant cleavage having occurred before complete binding

of the ATP. Preliminary results indicated that the equivalent rate is about 3–5 s^{-1} at 12 °C.

Unlabeled ATP Chase Experiments. These chase experiments were done for two reasons: first, to establish that most of the ATP is bound before significant completion of the turnover; second, to determine the rate at which bound ATP dissociates. For the first of these objectives, conditions which minimize the rate of ATP dissociation must be used because it is only possible to show that ATP is bound to the extent that it does not dissociate during completion of the turnover. Interaction of M*·ATP with actin results in ATP dissociation; high ionic strength, which reduces this interaction, correspondingly reduces the rate of dissociation (Sleep & Hutton, 1978). The reaction was started by the addition of a substoichiometric amount of labeled ATP to a myofibril suspension. After the time indicated on the abscissa, a chase solution (1 M KCl, 5 mM $MgCl_2$, 2 mM ATP, and 50 mM Tris, pH 8.5, at 25 °C) was added; the reaction was acid quenched 10 s later. The conditions of the chase (high pH and temperature) were chosen so as to make completion of the turnover unnecessary, because the equilibrium constant K_3 is sufficiently large for almost all the M*·ATP to go to M**·ADP·P_i (Taylor, 1977). This point was confirmed by the result being the same whether the chase solution was a pH 8.5, 25 °C, chase with a delay of 10 s before quenching or a pH 7, 0 °C, chase with a delay of 5 min to allow completion of the turnover (at the myosin rate). The results (Figure 1, curve labeled 1 M KCl) show that at 1 s, 95% of the nucleotide is committed to hydrolysis. The remaining 5% could either be in the medium or be bound but in exchange with medium ATP (be uncommitted), and this value is used as an upper limit on the amount of medium ATP.

Medium ATP could be present at 1 s either for kinetic reasons (that is, 1 s was insufficient time for complete binding) or for thermodynamic reasons, in which case it would provide a measure of the ATP binding constant. For acto-S1, the apparent second-order rate constant of ATP binding under the conditions of the myofibril experiments is 7×10^5 M^{-1} s^{-1}, and the excess concentration of myosin heads is 15 – 5 = 10 μM. The expected rate of binding is thus about 7 s^{-1}, or a half-life of 100 ms, and binding would be complete after 1 s (10 half-lives). This argument is not compelling as the K_m for ATP of fibers and myofibrils is larger than that of acto-S1, while the V_m is lower, which suggests that the apparent second-order rate constant of ATP binding might be less in the organized system (Sleep & Smith, 1981). However, the observation that the 1 M KCl chase curve decays at a very similar rate to the acid-quench curve provides a second argument against kinetic limitations on ATP binding, as shown by consideration of the following situation. If after 1 s, 90% of the ATP were bound and 10% free, then after 2 s, 99% of the ATP would be bound. If the ATP were present due to the slow rate of binding, the rate of decay of the 1 M KCl chase curve would be much faster than that of the acid-quench curve, and this is not observed. The limited number of 1 M KCl data points which are shown in Figure 1 do not in themselves allow a convincing estimate of the rate, but the results of other experimental runs all show that the decay of the 1 M KCl chase is only slightly faster than that of the acid-quench curve.

The rate of ATP release is needed to consider whether medium ATP is present at 1 s for thermodynamic reasons, and a value of 0.094 s^{-1} is deduced in the next section from the 0.1 M KCl ATP chase experiment shown in Figure 1. This rate together with the rate of ATP binding (7 s^{-1}) derived from acto-S1 rate constants suggests that at equilibrium 0.097/7 = 1.4% of the ATP would be in the medium (0.4% of the total nucleotide). As already mentioned, the rate of ATP binding may be slower for the organized system than for acto-S1, and, thus, it is likely that more than 0.4% of the nucleotide is in the form of medium ATP due to the equilibrium constant of ATP binding.

Low ionic strength maximizes interaction of actin and myosin states and for acto-S1 increases the rate of ATP dissociation. The results of a low ionic strength chase are given in Figure 1 (after mixing I = 0.035), and it can be seen that almost all the ATP of the acid-quench experiment remains after the chase; i.e., it was released into the medium. Higher ionic strength chases release progressively less of the bound ATP. For these chases, there was a 30-s interval between chase and acid quench to allow completion of the turnover. These results allow an estimate of the ratio of ATP released via actin interaction to ATP cleaved and released as products via actin interaction. The amplitude of the fast phase of the acid-quench curve (0.254) is the best estimate of the amount of ATP that would have been cleaved via actomyosin interaction. The amplitudes of the various ionic strength chases (0.24, 0.11, and 0.10 at I = 0.035, 0.085, and 0.13, respectively) provide an estimate of the proportion of ATP released, but the amplitude of the 1 M KCl chase (0.07) needs to be subtracted to allow for unbound ATP. The proportion of ATP released at I = 0.035 is (0.24 – 0.07)/(0.25 – 0.07) = 0.9, and the values at I = 0.085 and 0.13 are 0.26 and 0.20, respectively.

The rate of decay of bound ATP, k', is 0.45 s^{-1} in the range I = 0.035–0.175. The net forward rate from the myosin product state is $k'[(1 + K_3)/K_3]$, which, using the value for the equilibrium constant K_3 of 2.7 observed for S1 under these conditions, is equal to $0.45[(1 + 2.7)/2.7] = 0.61$ s^{-1}. For the chase at I = 0.035, a fraction 0.9 of that the ATP is released; that is, the ratio of bound nucleotide released as ATP to that released as products is 0.9/(2.7 + 0.1) = 0.32, and this equals ([M*·ATP] × the rate of ATP release)/([M**·ADP·P_i] × the rate of product release). The rate of ATP release is thus 0.61 s^{-1} × 2.7 × 0.9/2.8 = 0.53 s^{-1}. Corresponding rates of ATP release at I = 0.085 and 0.13 are 0.125 and 0.094 s^{-1}, respectively.

The rate of equilibration ($k_3 + k_{-3}$) of M*·ATP and M**·ADP·P_i is greater than the rate of ATP release, and as the equilibrium constant K_3 decreases with ionic strength (i.e., the proportion of M*·ATP increases), this could in part account for the greater amount of ATP release at low ionic strength. However, Taylor (1977) investigated the extent of equilibration of bound ATP and bound products by means of temperature and pH jumps during a single turnover. The extent decreased as the time between ATP addition and the jump increased, this process having a half-life of about 0.5 s (20 °C). Even on extrapolation to zero time, only about half the expected synthesis was observed, and thus it is not clear to what extent ATP synthesis should be allowed for when interpreting the myofibril experiments.

There is some scatter in the data points of the single experiment shown in Figure 1, and the difference between the 50 and 100 mM KCl chase curves appears small. The figure is representative, however, in that the consistent observations are that at least 80% of the ATP is released in the I = 0.035 chase and 20–35% in the I = 0.13 and 0.085 chases, the difference between the latter chases being small but reproducible. The accuracy of the measurement of the off rate of ATP at the higher ionic strengths is not good because the 1-s chase at 100 mM KCl results in 10% of the ATP being present at quench time, while with a 1 M KCl chase, 5% is present.

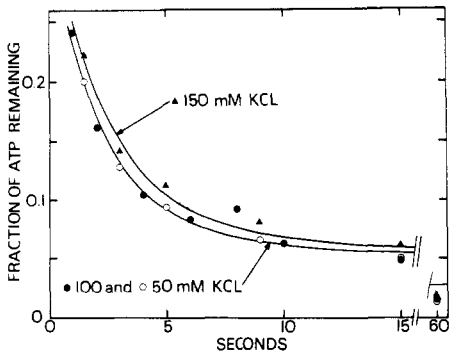


FIGURE 3: Effect of ionic strength on acid-quenched single turnovers. Experiments were conducted like those for the acid-quench curve of Figure 1: (○) 50 mM KCl; (●) 100 mM KCl; (▲) 150 mM KCl. The lower line is a fit to the 50 and 100 mM KCl data and has a rate of 0.45 s^{-1}; the upper line is a fit to the 150 mM KCl data and has a rate of 0.4 s^{-1}.

It has to be assumed that the 5% represents medium ATP, and, thus, this is subtracted, halving the amount apparently released. As it is possible that some release occurs even at 1 M KCl, the rate of ATP release at 100 mM KCl could be as much as 2 times greater than the present estimate.

Ionic Strength Dependence of the Turnover Rate. The rate of binding of M**·ADP·P_i to actin in solution is very dependent on ionic strength, being slower at high ionic strength, and it is of interest to know whether this dependence is preserved in myofibrils. The results are shown in Figure 3. One line corresponds to the experimental points at 50 and 100 mM KCl and has a rate of 0.45 s^{-1}; the other corresponds to the experiment at 150 mM KCl and has a rate of 0.4 s^{-1}. At the time of the original experiments, an example of which is shown in Figure 3, the decay curves were not measured at very low ionic strength because of its effect on interfilament spacing. The decay rate at $I = 0.035$ was later measured because of its relevance to interpretation of the low ionic strength chase experiments: the rate was found to be similar to that at higher ionic strengths. The lack of dependence of the rate of decay on ionic strength contrasts sharply with the association reaction in solution (White & Taylor, 1976). An interpretation in terms of the simple Lymn–Taylor scheme involves either the effective actin concentration changing with ionic strength or there being a fortuitous balance of two effects of low ionic strength: acceleration of actin binding to M**·ADP·P_i and inhibition of the rate of ATP decay by titration of M*·ATP to AM·ATP. As discussed later, the finding is more plausibly interpreted in terms of a model of acto-S1 ATPase in which the rate of ATP decay is controlled by a first-order step and not an actin binding step.

Diffusion of ATP into Myofibrils. Diffusion of ATP into myofibrils would be expected to be fast on the time scale of the experiments reported here. This point can, however, be readily checked because variation of the ratio of myosin heads to ATP molecules would be predicted to have no effect on the time course of ATP decay. At high ratios, only the myosin heads on the periphery of the myofibril would partake in the reaction, and thus, effects resulting from diffusion times into the fiber would be reduced. In an experiment to test this, the head concentration was kept constant at 20 μM (after mixing), and the ATP concentration varied over a range from 10 to 1 μM. No significant difference in the decay of ATP was apparent. A related question is whether the concentration of unlabeled ATP in the chase was adequate. The concentration in the standard chase was 2 mM, but no difference was found between high ionic strength (1 M KCl) chase solutions in the range 1–10 mM MgATP.

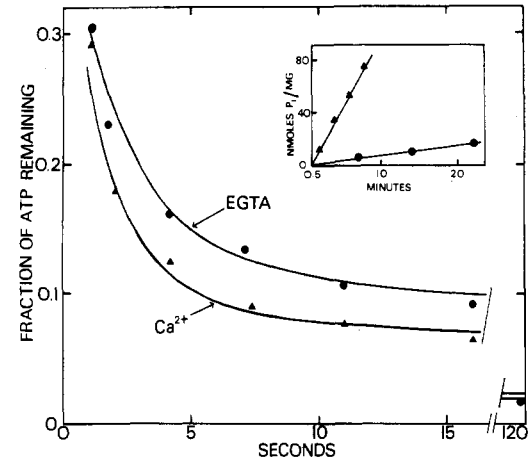


FIGURE 4: Effect of Ca^{2+} concentration of acid-quenched single turnovers. Experiments were conducted like those for the acid-quench curve of Figure 1: (▲) 100 μM $CaCl_2$; (●) 2 mM EGTA. The curves fitted to the Ca^{2+} and EGTA points have rates of 0.55 and 0.45 s^{-1}, respectively. Inset: Dependence of steady-state ATPase rate on Ca^{2+} concentration. The ordinate is a measure of the increase in P_i over that existing 30 s after addition of ATP. Reaction conditions: 100 mM KCl, 20 mM imidazole, 3 mM $MgCl_2$, and 1 mM [γ-^{32}P]ATP; (▲) 100 μM $CaCl_2$; (●) 2 mM EGTA.

Single Turnovers and Ca^{2+} Control. Acid-quenched single turnovers in the presence and absence of Ca^{2+} are shown in Figure 4, and it can be seen that the absence of Ca^{2+} makes little difference. The inset in Figure 4 shows steady-state ATPase measurements. The turnover rate per myosin head is 0.072 s^{-1} in the presence of Ca^{2+} and 0.008 s^{-1} in its absence. The control ratio of 9 (0.072/0.008) is roughly that expected because the rate in the absence of Ca^{2+} corresponds to the S1 rate at 0 °C. The rate of the fast phase of the fitted curve to the Ca^{2+} points is 0.55 s^{-1}, and that to the EGTA points is 0.45 s^{-1}. There was some variation from preparation to preparation; for some, the presence of Ca^{2+} made no difference to the decay rate, while for others, it increased the rate by up to 50%. The fraction of ATP existing throughout the time course is slightly less in the presence than in the absence of Ca^{2+}, and this is a consistent observation. The cause is unknown, but S1 single-turnover experiments (0 °C, 10 mM KCl, pH 7) done in another context showed a similar small effect of Ca^{2+} on ATP levels. The present observation on the lack of Ca^{2+} control on single turnovers is consistent with a similar finding for single turnovers of regulated acto-S1 (Taylor, 1979). These results can readily be accounted for by models of thin filament cooperativity (Weber & Murray, 1973) in which myosin heads bound in rigor to actin sites keep the neighboring sites "switched on" even in the absence of Ca^{2+}.

Acto-S1 Unlabeled ATP Chase Experiments. To interpret the results of myofibril experiments quantitatively in terms of the acto-S1 scheme, it was necessary to measure certain rate and equilibrium constants under the conditions of the myofibril experiments (100 mM KCl, 3 mM $MgCl_2$, and 20 mM imidazole, pH 7, 0 °C). The equilibrium constant of the hydrolysis step K_3 (eq 1) was measured by the method of Bagshaw & Trentham (1973) and found to be 2.7. Experiments of the type reported by Sleep & Hutton (1978) were performed to determine the ratio of apparent second-order rate constants of actin binding to M**·ADP·P_i and M*·ATP (k_4^a/k_{-2}^a). A substoichiometric amount of [γ-^{32}P]ATP was added to S1 followed 5 s later by actin and unlabeled ATP, and the fate of the bound, labeled ATP was determined. At actin concentrations in the range 10–100 μM, 3.5% of the bound ATP was released by actin. The small extent of release and the lack of dependence upon actin concentration are

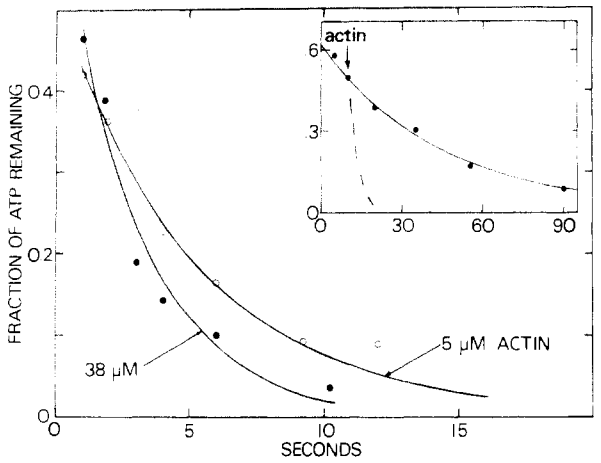


FIGURE 5: Time courses of decay of bound ATP. To 100 μL of S1 (6 μM) was added 20 μL of [γ-^{32}P]ATP (9 μM), and after 10 s had elapsed, actin was added [0.225 mL of 60 μM or 1 mL of 6 μM; final free actin concentrations [[actin] − ([S1] − [ATP])] were 38 and 5 μM]. Such reaction mixtures were acid quenched at a series of times after actin addition. The lines are single exponentials fitted by a weighted least-squares procedure: for the 38 μM points, the curve is $y = 0.66 \exp(-0.345t)$, and for the 5 μM points, $y = 0.51 \exp(-0.19t)$. Inset: Decay in the absence of actin. The curve is $y = 0.62 \exp(-0.022t)$. The dashed curve represents a double-mixing experiment and shows the accelerated decay of ATP upon addition of actin at 10 s. Reaction conditions: 10 mM KCl, 1 mM $MgCl_2$, and 10 mM imidazole, pH 7, 0 °C.

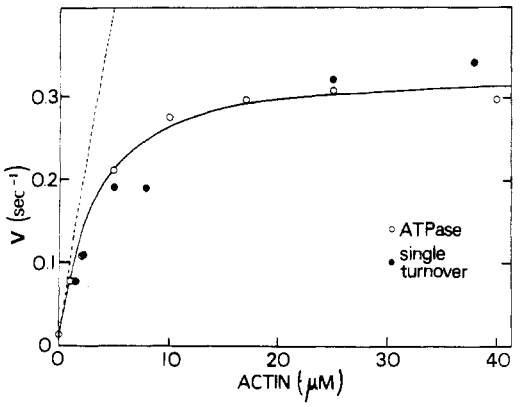


FIGURE 6: Comparison of the actin dependence of the steady-state ATPase and the rate of decay of bound ATP. (○) Steady-state ATPase rate; (●) decay of bound ATP. The solid line is a hyperbola ($K_m = 3$ μM, $V_m = 0.34$ s^{-1}) fitted to the steady-state ATPase data. Reaction conditions were as for Figure 5; for the steady-state ATPase rate, the S1 concentration was 1 μM, and the ATP concentration was 100 μM. The dashed line is the prediction of a simple Lymn–Taylor scheme ($k_3 = 2$ s^{-1}, K_2 and $k_{\pm d} = 0$).

consistent with the previously reported large decrease in the extent of ATP release at 40 mM KCl compared to 5 mM KCl (13 and 36%, respectively; values extrapolated to zero actin concentration; Sleep & Hutton, 1978). The ratio of bound nucleotides released as ATP to those released as products is $k_{-2}^a/K_3k_4^a$. The observed release of 3.5% of the bound ATP corresponds to $3.5/(1.0 + 2.7) = 0.95$ of the bound nucleotide; therefore, $k_{-2}^a/k_4^a = [0.95/(100 - 0.95)]2.7 \approx 0.025$.

Acto-S1 Single-Turnover Experiments. These experiments were done at 0 °C to allow mixing to be done in a stirred test tube instead of a quench flow machine, and at low ionic strength to allow use of an actin concentration many times the K_m for actin. The experiment was done both in the form of a direct single turnover (ATP added to acto-S1) and also in the form of a double-mixing experiment (ATP added to S1 and then actin added). The results obtained by the two methods were similar although the maximum rate of ATP decay was 10–15% slower with the direct method. The double-mixing method avoids uncertainty about unbound ATP at the beginning of the decay, and for this reason, it is the preferred method.

The inset of Figure 5 shows an S1 single turnover. The fraction of ATP extrapolates back to 0.62 at zero time, and, thus, the equilibrium constant K_3 under these conditions is $0.38/0.62 = 0.61$. The double-mixing experiment is represented diagrammatically in the inset. ATP is added to S1, and the bound ATP slowly decays. After 10 s, actin is added, and the accelerated decay of ATP is observed. This is plotted in the main part of Figure 5. The lines are single exponentials fitted by a weighted least-squares procedure. Even for the simplest model, the theoretical curve is not a single exponential but the sum of two exponentials. However, the quality of the data does not allow fitting of this more complicated function. The decay curve at 38 μM actin extrapolates to a slightly higher point than that at 5 μM actin; that is, high actin concentration appears to induce a burst of ATP synthesis. Although not investigated in detail, this observation was reproducible. It is for this reason that the zero-time point (i.e., the acid quench 10 s after ATP addition) is not included in the curve fitting.

The steady-state ATPase rate is plotted against actin concentration in Figure 6. The line is a hyperbola with a K_m of 3 μM and a V_m of 0.34 s^{-1}. The rates of ATP decay in acto-S1 single-turnover experiments are included in this figure. Although there is some scatter, the latter points correspond reasonably with the hyperbola; in particular, the rate of decay at the highest actin concentration is only 0.34 s^{-1}.

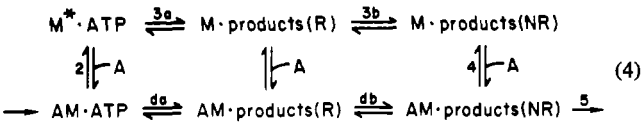
Discussion

The acto-S1 experiments will be considered first as they facilitate understanding of the myofibril experiments; in practice, it was the myofibril experiments which led to the acto-S1 experiments.

The rate of ATP decay during a single turnover and the steady-state ATPase rate of acto-S1 are reasonably similar over a range of actin concentrations up to 10 times the K_m (Figure 6), which suggests that both processes are controlled by the same enzymatic step. This is not the case for the simple Lymn–Taylor scheme, for which at high actin concentration the steady-state rate is controlled by the product-release step k_5 and the rate of ATP decay by the hydrolysis step k_3 (eq 2). Eisenberg and collaborators have shown that the explanation does not lie in the fortuitous equality of k_3 and k_5 (Stein et al., 1979). The first modification of the Lymn–Taylor scheme is to allow step 2 to be reversible (Lymn, 1974; Taylor, 1979) such that at high actin concentrations a significant proportion of bound ATP is in the form AM·ATP. In one form of this model at high actin concentrations, the rate of the hydrolysis step k_3, inhibited by the term $1/(1 + K_2[A])$, governs both the steady-state ATPase rate and the rate of ATP decay, which accounts for the observed similarity of the two rates. This model is not satisfactory because the dependence of the rates on actin concentration deviates far more from a hyperbola than is consistent with the data.

The data can be accounted for in a simple manner by the original refractory-state scheme (eq 3; Chock et al., 1976). This model has the desired feature of the steady-state ATPase rate and the bound ATP decay rate being controlled by the same steps (at low actin concentration by $k_4^a[A]$, where k_4^a is the apparent second-order rate constant of actin binding to M**·ADP·P_i, and at high actin concentration by k_{3b}) and also predicts the observed hyperbolic dependence of rate on actin

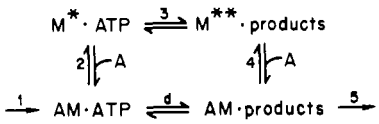
concentration. However, this model is unable to account for other acto-S1 data and has accordingly been modified by the inclusion of a nondissociating pathway (Stein et al., 1979).



(4)

In this model, there are equivalent ATP, refractory (R) and nonrefractory (NR) states for actin-bound and free myosin, and the binding of actin does not change the transition rates between these states (i.e., $k_{3a} = k_{da}$ and $k_{3b} = k_{db}$). The rate-limiting step at saturating actin concentration is that between AM·products(R) and AM·products(NR) and at low actin concentration is actin binding to M·products(R) and M·products(NR). This scheme preserves the features of the simple refractory-state scheme which accounted for the results of the acto-S1 single-turnover experiments.

Taylor (unpublished experiments) has found evidence in favor of a model which is the simple Lymn–Taylor scheme with the addition of a nondissociating pathway (eq 5). At



(5)

low actin concentrations, the actin binding step controls both the steady-state ATPase and the rate of ATP decay (the rate is k_4^a[A]). At saturating concentrations of actin, dissociation does not occur, and the steady-state ATPase rate is controlled by the hydrolysis step (k_d). In the single-turnover experiment at high actin concentrations, upon addition of actin, the state M*·ATP is immediately converted to AM·ATP, and the decay of this ATP is controlled by the hydrolysis rate k_d. The same first-order step thus controls the rates of ATPase and the decay of bound ATP. With an appropriate assignment of rate constants, the model predicts an approximately hyperbolic dependence of rate on actin concentration.

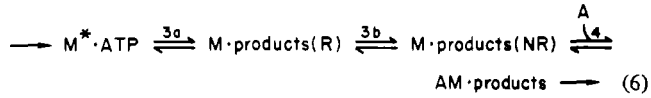
Under Results, it was mentioned that a consistent finding was that there appeared to be a synthesis of ATP prior to loss of ATP through hydrolysis. The modified refractory-state scheme of Stein et al. (1979) would predict such behavior, provided the equilibrium constant K_{da} were less than K_{3a} (eq 4). On addition of actin, the myosin states M*·ATP and M·products(R) would rapidly bind to actin, and because the subsequent step (k_{db}) is the rate-limiting step, the states AM·ATP and AM·products(R) would reequilibrate at $k_{da} + k_{-da}$ prior to decay at k_{db}. In the case of the modified Lymn–Taylor scheme (eq 5), it is more difficult to find a set of rate constants which leads to synthesis because it is the hydrolysis step k_d and not the subsequent step k_5 that is rate limiting. When M**·products binds to actin, product release will tend to occur rather than ATP synthesis.

Myofibril Single Turnovers. In a single turnover of ATP by myofibrils, about 70% is observed to be split rapidly and most of the remaining 30% at a rate of about 0.45 s^{-1}. The general interpretation is that the 70% corresponds to equilibration of the bound ATP states (M*·ATP and AM·ATP) and bound product states (M**·ADP·P_i and AM·ADP·P_i) and that the slow, observed phases correspond to the rate of product states proceeding toward product release. These experiments will first be considered on the basis of a simple Lymn–Taylor scheme.

The rate of the observed decay of bound ATP (k') is 0.45 s^{-1}, which is much less than the rate at which the ATP cleavage step equilibrates [$k_3 + k_{-3} = 8$ s^{-1}; extrapolated from Johnson & Taylor (1978)]. The observation that 70% of the ATP is split in the first second shows directly that the rate k' is not controlled by the hydrolysis rate. Since most of the ATP is bound early in the reaction, the rate k' can only correspond to the net rate of M**·ADP·P_i binding to actin and the reaction proceeding toward product release. On this model, ATP binds to a myosin head in rigor linkage, and the head dissociates, forming M*·ATP. Equilibrium is rapidly established between M*·ATP and M**·ADP·P_i, and the observed phase of ATP decay is due to M**·ADP·P_i binding to actin with maintenance of the M*·ATP to M**·ADP·P_i equilibrium ratio. In solution, M**·ADP·P_i binding to actin is a second-order step, and the net forward rate of this step can be described by an apparent second-order rate constant k_4^a (White & Taylor, 1976); that is, the net rate of actin binding is k_4^a[A]. For myofibrils, because of the ordered lattice, cross-bridge binding is better described as first order, and k_4' is used to describe the rate of this step (k_{-2}' is used correspondingly for actin binding to M*·ATP). These rate constants refer to the processes of actin binding and committing ATP or products to release. On the Lymn–Taylor scheme, the experimentally observed k' is equal to $k_4'[K_3/(1 + K_3)]$. The rate k' was found to be independent of ionic strength, whereas in solution, the step of actin binding to M**·ADP·P_i is very dependent on ionic strength. On the simple Lymn–Taylor scheme, this difference would have to be ascribed to some unknown difference in the nature of the biochemical step in solution and in a myofibril.

The unlabeled ATP chase experiments were interpreted within the Lymn–Taylor scheme under Results. The rate of ATP dissociation was quite dependent on ionic strength, which suggests that the lack of ionic strength dependence of k' is not due to some general effect of the lattice. At 100 mM KCl, the best estimate of k_{-2}'/k_4' was 0.15, which is to be compared with the ratio of apparent second-order rate constants k_{-2}^a/k_4^a of 0.025 for acto-S1 under similar conditions (0 °C, 100 mM KCl). If cross-bridge binding in a myofibril could be described by the apparent second-order rate constant determined for acto-S1, together with an effective actin concentration, then the ratio of first-order rates in a myofibril (k_{-2}'/k_4') would match the ratio of apparent second-order rate constants (k_{-2}^a/k_4^a). The observed difference could be indicative of the model being incorrect, or it could reflect mechanical constraints accelerating the reverse rate compared to the forward rate.

The myofibril data will now be considered in terms of modified Lymn–Taylor schemes. The original refractory-state scheme (eq 6; Chock et al., 1976) readily accounts for the data.



(6)

The net forward rate of step 4 is much greater than k_{-3b}, and thus, k' corresponds to k_{3b}. The maximum rate of acto-S1 at saturating actin concentration V_m is also controlled by k_{3b}. A definite prediction of the model is thus that k' should equal V_m of acto-S1, which it does reasonably satisfactorily at both 0 and 12 °C [$k' = 0.45$ and 3–5 s^{-1} at 0 and 12 °C; V_m = 0.25–0.45 s^{-1} at 0 °C (Eisenberg & Kielley, 1973; Marston, 1978); $V_m = 5$ s^{-1} at 15 °C (Chock et al., 1976)]. The observed lack of ionic strength dependence of k' is now expected because k' is not controlled by an actin binding step but by k_{3b}. This also controls the acto-S1 V_m, which has limited ionic strength dependence (Eisenberg & Moos, 1968). In contrast, the unlabeled ATP chase experiment is dependent on ionic strength because the result depends on a competition between an actin binding step (A + M*·ATP, ionic strength dependent)

and a first-order step (R → NR, independent of ionic strength).

For the Lymn–Taylor scheme, the dissociated states (M*·ATP and M**·ADP·P_i) are in equilibrium, and the result of the chase experiment is dependent on the ratio of first-order rates k_{-2}'/k_4'. The equivalent ratio of apparent second-order rates was measured in solution, and the ratios do not agree. For the refractory-state scheme, the R → NR step is rate limiting, and thus the dissociated states are not in equilibrium: the myofibril result could only be predicted from acto-S1 rate constants if the effective actin concentration of myofibrils was known.

Schemes which include a nondissociating pathway must now be considered. The refractory-state scheme of this type (Stein et al., 1979) preserved the useful features of the original refractory-state scheme. The rate of ATP decay at high actin concentrations is still controlled by a first-order step and not one involving actin binding, and thus the lack of ionic strength dependence is expected. Similarly, the rate of ATP decay would still be expected to be equal to the acto-S1 V_m.

Finally, there is the simple nondissociating pathway model proposed by Taylor. If the effective actin concentration of a muscle were sufficiently low such that most of the heads were dissociated, then its predictions would be similar to the simple Lymn–Taylor scheme already considered. If the effective actin concentration were high such that most heads were associated, then ATP decay would be controlled by a first-order step (the hydrolysis step k_d) and would thus be predicted to be independent of ionic strength. In the models previously considered, a slow step followed the hydrolysis step, and this accounted for the observed decay of ATP in two phases (70% fast, equilibration of hydrolysis step; 30% slow, posthydrolysis step). In the nondissociating model of Taylor, there is not a slow step following hydrolysis, and only a single phase of ATP decay would be predicted. This model is not able to account for the results of the myofibril single-turnover experiments in a simple manner.

The rate k' is also relevant to the question of the percentage of heads associated with actin during an isometric contraction. If it is assumed initially that the only pathway involves an obligatory dissociation at AM·ATP, then the percentage of heads that are dissociated can be estimated from k' and the steady-state ATPase rate per head. Models in which nondissociating pathways are important will give higher predictions for the percentage of heads bound. A reliable value for the steady-state ATPase rate for an isometric contraction cannot be obtained for myofibrils due to the problem of contraction to short sarcomere lengths at which there is incomplete overlap of the filaments. The data of Figure 4 correspond to k_{ss} = 0.072 s^{-1}, while Marston & Tregear (1974) obtained values of 0.13 s^{-1} for isometric fibers and 0.15 s^{-1} for myofibrils under conditions which prevented shortening. A steady-state rate of 0.072 s^{-1} would be interpreted in terms of their being a 0.086-s^{-1} step in the associated part of the scheme and a 0.45-s^{-1} step in the dissociated part. These values correspond to the system being 84% associated during a maximal isometric contraction. Marston's rate of 0.15 s^{-1} would correspond to a rate of 0.225 s^{-1} in the associated part of the scheme and 67% of the heads being associated. These estimates are surprisingly high because the tension, and more importantly the stiffness, of rabbit fibers decreases considerably with temperature (Kawai, 1979), and this can be most simply interpreted in terms of fewer heads than maximal being bound.

Thermodynamics of the Steps from AM to A + M·ATP.* As has been discussed in detail by Eisenberg & Hill (1978), for S1 the major part of the free-energy change of ATP hydrolysis occurs in the binding step (M + ATP → M*·ATP). For acto-S1, the binding of ATP results in the dissociation of actin because the binding of the two ligands is antagonistic, and as a result, much of the energy is conserved. In many models of contraction, steps which result in force-producing states occur in that part of the scheme from AM·ADP·P_i to AM + products, where a large part of the basic free-energy change of ATP hydrolysis is thought to occur (White & Taylor, 1976). In such schemes, basic free-energy changes which occur between AM + ATP and A + M*·ATP may be wasted, in the sense that this energy cannot be converted to work. It is thus important to establish what fraction of the total basic free-energy change of ATP hydrolysis occurs in these steps. Estimates can be obtained either kinetically or thermodynamically. The kinetic method is to consider the ratio of rates of ATP binding and dissociation. Sleep & Hutton (1978) compared the estimates of the free-energy change from AM + ATP to A + M*·ATP derived from the binding constants of actin and ATP to S1 and from measurements of apparent second-order rate constants of actin binding to M*·ATP and of ATP binding to AM. On a simple model (eq 2) in which there is only a single AM·ATP state, the former rate is $k_{-1}K_2$, and the latter is k_1, which in combination gives K_1/K_2. For acto-S1, the kinetic method was found to give values as close as could be expected to the thermodynamic method, allowing for the limited accuracy of the latter measurements. As discussed earlier, the rate of ATP release k_{-2}' for myofibrils is 0.094 s^{-1} at I = 0.13, and this represents $k_{-1}K_2$. Unfortunately, there are no direct measurements of k_1 for myofibrils, and the value for acto-S1 [7 × 10^5 M^{-1} s^{-1}; extrapolation of the data of White & Taylor (1976)] will have to be used. At a physiological concentration of ATP (4 mM), the basic equilibrium constant from AM + ATP to A + M*·ATP is thus 4 × 10^{-3} M × 7 × 10^5 M^{-1} s^{-1}/0.09 s^{-1} = 3 × 10^4.

In the case of the thermodynamic method, there are no assumptions involved, but it only gives a lower limit. At 1 s, 25% of the nucleotide is ATP and ≥5% is not bound. The ratio bound ATP/medium ATP is thus ≥20/5 = 4. The association constant is (bound ATP/medium ATP)(1/myosin heads): the myosin head concentration is 15 − 5 = 10 μM, giving K_{assoc} ≥ 4 × 10^5 and the basic equilibrium constant for ATP binding ≥1600. The basic equilibrium constant of ATP hydrolysis is about 10^{10} (4 mM ATP, 30 μM ADP, and 1 mM P_i), and ATP binding is associated with at least 30% (thermodynamic estimate) and possibly as much as 40% (kinetic estimate) of the total free-energy change. This result suggests that AM·ATP might be an important force-generating state in the contractile cycle.

Acknowledgments

I thank Dr. R. M. Simmons for valuable comments on the manuscript.

References

Arata, T., & Tonomura, Y. (1976) *J. Biochem. (Tokyo) 80*, 1353.

Bagshaw, C. R., & Trentham, D. R. (1973) *Biochem. J. 133*, 323.

Bagshaw, C. R., & Trentham, D. R. (1973) *Biochem. J. 133*, 323.

Bagshaw, C. R., & Trentham, D. R. (1974) *Biochem. J. 161*, 331.

Chock, S. P., Chock, P. B., & Eisenberg, E. (1976) *Biochemistry 15*, 3244.

Cooke, R., & Francks, K. (1980) *Biochemistry 19*, 2265.
Drabikowski, W., & Gergely, J. (1964) *Biochem. Muscle Contract., Proc. Symp.*, 125.
Eisenberg, E., & Moos, C. (1968) *Biochemistry 7*, 1486.
Eisenberg, E., & Kielley, W. W. (1973) *Cold Spring Harbor* Symp. *Quant. Biol. 37*, 145.
Eisenberg, E., & Hill, T. L. (1978) *Prog. Biophys. Mol. Biol. 33*, 55.
Eisenberg, E., Dobkin, L., & Kielley, W. W. (1972) *Proc. Natl. Acad. Sci. U.S.A. 69*, 667.
Greene, L. E., & Eisenberg, E. (1980) *J. Biol. Chem. 255*, 543.
Huxley, H. E. (1969) *Science (Washington, D.C.) 164*, 1356.
Johnson, K. A., & Taylor, E. W. (1978) *Biochemistry 17*, 3432.
Kawai, M. (1979) in *Crossbridge Mechanism in Muscle Contraction* (Sugi, H., & Pollack, G. H., Eds.) University of Tokyo Press, Tokyo.
Lymn, R. W. (1974) *J. Theor. Biol. 43*, 313.
Lymn, R. W., & Taylor, E. W. (1970) *Biochemistry 9*, 2975.
Lymn, R. W., & Taylor, E. W. (1971) *Biochemistry 10*, 4617.
Marston, S. (1978) *FEBS Lett. 92*, 147.
Marston, S. B., & Tregear, R. T. (1974) *Biochim. Biophys. Acta 333*, 581.
Perry, S. V. (1955) *Methods Enzymol. 2*, 582.
Perry, S. V., & Grey, T. C. (1956) *Biochem. J. 64*, 184.
Schendel, P. F., & Wells, R. D. (1973) *J. Biol. Chem. 248*, 8319.
Sleep, J. A., & Taylor, E. W. (1976) *Biochemistry 15*, 5813.
Sleep, J. A., & Hutton, R. L. (1978) *Biochemistry 17*, 5423.
Sleep, J. A., & Smith, S. J. (1981) *Curr. Top. Bioenerg.* (in press).
Stein, L., Schwarz, R. P., Chock, P. B., & Eisenberg, E. (1979) *Biochemistry 18*, 3895.
Sutoh, K., & Harrington, W. F. (1977) *Biochemistry 16*, 2441.
Taylor, E. W. (1977) *Biochemistry 16*, 732.
Taylor, E. W. (1979) *CRC Crit. Rev. Biochem. 6*, 103.
Thomas, D. D., Seidel, J. C., & Gergely, J. (1978) *Biophys. J. 21*, 43a.
Weber, A., & Murray, J. M. (1973) *Physiol. Rev. 53*, 612.
Weeds, A. G., & Taylor, R. S. (1975) *Nature (London) 257*, 54.
White, H. D. (1977) *Biophys. J. 17*, 40a.
White, H. D., & Taylor, E. W. (1976) *Biochemistry 15*, 5818.

Excitation Energy Transfer Studies on the Proximity between SH_1 and the Adenosinetriphosphatase Site in Myosin Subfragment 1†

Terence Tao* and Mark Lamkin

ABSTRACT: Excitation energy transfer studies were carried out to determine the distance between the adenosinetriphosphatase (ATPase) site and a unique "fast-reacting" sulfhydryl (referred to as SH_1) in myosin subfragment 1. The fluorescent moiety of the probe *N*-(iodoacetyl)-*N'*-(5-sulfo-1-naphthyl)ethylenediamine was used as the donor attached at SH_1. The chromophoric nucleotide analogue 2'(3')-*O*-(2,4,6-trinitrophenyl)adenosine 5'-diphosphate was used as the acceptor noncovalently bound at the ATPase site. The energy transfer efficiency was found to be 56% by measuring the decrease in donor fluorescence lifetime. The critical transfer distance, $R_0(^2/_3)$, was determined to be 40.3 Å. Since both donor and acceptor are likely to be rigidly attached, a statistical interpretation of the data was applied [Hillel, Z., & Wu, C.-W. (1976) *Biochemistry 15*, 2105] to determine distances. The method yielded the following conclusions: most probable distance = 38.7 Å; maximum possible distance = 52 Å; 10% probability for the distance to be less than 20 Å; 3% probability to be less than 15 Å. It may be concluded that despite the great influence that the two sites exert on each other, it is not likely that SH_1 interacts directly with the ATPase site in myosin subfragment 1. This conclusion is in agreement with the findings of Wiedner et al. [Wiedner, H., Wetzel, R., & Eckstein, F. (1978) *J. Biol. Chem. 253*, 2763] and Botts et al. [Botts, J., Ue, K., Hozumi, T., & Samet, J. (1979) *Biochemistry 18*, 5157].

It is well-known that myosin subfragment 1 (S1)[1] (prepared from myosin by proteolytic digestion) retains its capacity to hydrolyze ATP and contains a pair of "fast-reacting" sulfhydryls (referred to as SH_1 and SH_2). Much current work is directed toward relating the conformational states of S1 with steps in the hydrolysis cycle. In this respect, it is of importance to find methods that are capable of defining the conformation of S1. One such method is based on singlet–singlet excitation energy transfer [see reviews by Fairclough & Cantor (1978) and Stryer (1978)], which has been used by a number of workers to measure distances between sites located either within or between contractile proteins (Marsh & Lowey, 1980; Takashi, 1979; Miki & Mihashi, 1978; Miki, 1979; Haugland, 1975). Of particular interest is the distance separating the ATPase site from SH_1 and SH_2, for it is well-known that modification of SH_1 and/or SH_2 affects the ATPase activities of myosin (Sekine & Kielley, 1964; Yamaguchi & Sekine, 1966). Conversely, the reactivity of SH_2 is affected by the presence of a bound nucleotide at the ATPase site (Yamaguchi & Sekine, 1966). Furthermore, recent work found that

† From the Department of Muscle Research, Boston Biomedical Research Institute, Boston, Massachusetts 02114. *Received December 30, 1980.* This work was supported by Grant AM-21673 from the National Institutes of Health.

[1] Abbreviations used: S1, myosin subfragment 1; 1,5-IAEDANS, *N*-(iodoacetyl)-*N'*-(5-sulfo-1-naphthyl)ethylenediamine; AEDANS-S1, S1 labeled with 1,5-IAEDANS; TNP-ATP, TNP-ADP, and TNP-AMP, 2'(3')-*O*-(2,4,6-trinitrophenyl)adenosine 5'-triphosphate, -diphosphate, and -monophosphate, respectively; Hepes, *N*-2-(hydroxyethyl)-piperazine-*N'*-2-ethanesulfonic acid; $NaDodSO_4$, sodium dodecyl sulfate.

0006-2960/81/0420-5051$01.25/0