

Reaction Mechanism of the Magnesium Ion-Dependent Adenosine Triphosphatase of Frog Muscle Myosin and Subfragment 1

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The Mg^{2+} -dependent ATPase (adenosine 5'-triphosphatase) mechanism of myosin and subfragment 1 prepared from frog leg muscle was investigated by transient kinetic techniques. The results show that in general terms the mechanism is similar to that of the rabbit skeletal-muscle myosin ATPase. During subfragment-1 ATPase activity at 0–5°C, pH 7.0 and $f_0.15$, the predominant component of the steady-state intermediate is a subfragment-1-products complex (E·ADP·P_i). Binary subfragment-1-ATP (E·ATP) and subfragment-1-ADP (E·ADP) complexes are the other main components of the steady-state intermediate, the relative concentrations of the three components E·ATP, E·ADP·P_i and E·ADP being 5.5:92.5:2.0 respectively. The frog myosin ATPase mechanism is distinguished from that of the rabbit at 0–5°C by the low steady-state concentrations of E·ATP and E·ADP relative to that of E·ADP·P_i and can be described by:

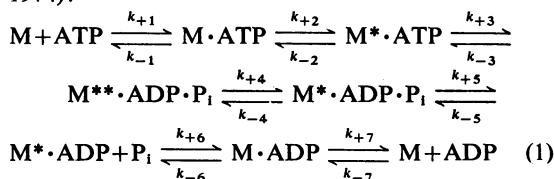


In the above conditions successive forward rate constants have values: k'_{+1} , $1.1 \times 10^5 M^{-1} \cdot s^{-1}$; $k'_{+2} > 5 s^{-1}$; k'_{+3} , $0.011 s^{-1}$; k'_{+4} , $0.5 s^{-1}$; k'_{-1} is probably $< 0.006 s^{-1}$. The observed second-order rate constants of the association of actin to subfragment 1 and of ATP-induced dissociation of the actin-subfragment-1 complex are $5.5 \times 10^4 M^{-1} \cdot s^{-1}$ and $7.4 \times 10^5 M^{-1} \cdot s^{-1}$ respectively at 2–5°C and pH 7.0. The physiological implications of these results are discussed.

Comparison of biochemical results obtained *in vitro* with the physiological studies on living muscle are hampered by differences in experimental conditions and species. To facilitate such a comparison the present paper describes the results of transient kinetic experiments in which the mechanism of frog myosin and subfragment-1 Mg^{2+} -dependent ATPase was examined under conditions as similar as is practical to those of physiological work *in vivo*.

A proposed mechanism for the Mg^{2+} -dependent ATPase of myosin isolated from rabbit skeletal

muscle is given in eqn. (1) (Bagshaw & Trentham, 1974):



in which k_{+i} and k_{-i} are the forward and reverse rate constants for the *i*th step respectively. The asterisks are used to distinguish isomeric intermediates as well as to denote an enhancement of the protein fluorescence over that of the native protein.

Salient features of the Mg^{2+} -dependent ATPase mechanism that have been established for rabbit myosin and subfragment 1 are as follows. (a) The predominant steady-state intermediate at 20°C is

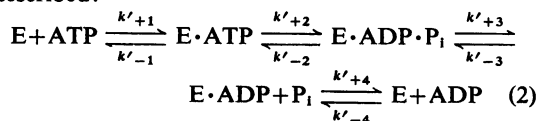
Abbreviations used: ATPase, adenosine 5'-triphosphatase; thioITP, 6-mercapto-9-β-D-ribofuranosylpurine 5'-triphosphate.

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the myosin-products complex, $M^{**}\cdot ADP\cdot P_i$. This conclusion follows primarily from Lymn & Taylor's (1971) experiments in which molar excess of ATP was mixed with protein and the reaction mixture quenched by acid at various times. They found that the rate of P_i formation was biphasic. The amount of P_i formed in the transient phase corresponded to 0.8 mol of P_i /mol of myosin subfragment-1 heads. (b) The ATP-cleavage step, which ^{18}O experiments suggest is simply ATP hydrolysis (Trentham, 1977), is readily reversible with a value of $K_3 (=k_{+3}/k_{-3})$ of 9 (Bagshaw & Trentham, 1973). (c) The binary complex $M^{**}\cdot ADP$ is the same complex as that formed when ADP is mixed directly with the protein. At 20°C the rate constant controlling ADP dissociation, k_{+6} , is severalfold higher than the catalytic-centre activity, $k_{cat.}$, whereas at 5°C k_{+6} is comparable with $k_{cat.}$. Consequently at 5°C $M^{**}\cdot ADP$ forms a significant fraction of the steady-state intermediate (Bagshaw & Trentham, 1974). These properties are described in more detail by Trentham *et al.* (1976).

The time course of ATP hydrolysis by frog myosin and subfragment 1 has been studied by quenched-flow techniques (Lymn & Taylor, 1970) and stopped-flow spectrophotometry (Bagshaw *et al.*, 1972). The frog myosin and subfragment-1 preparations described in the preceding paper (Ferenczi *et al.*, 1978) provide sufficient protein for the above properties to be examined and hence to establish the extent to which eqn. (1) is a satisfactory description of the frog myosin Mg^{2+} -dependent ATPase mechanism.

Although the results indicate a qualitative agreement with eqn. (1), frog myosin and its subfragment 1 do not exhibit a large fluorescence enhancement of the native protein on binding of ATP or ADP. This and the greater lability of the frog proteins prevented us from carrying out experiments that might show the two-step binding of ATP, ADP and P_i (Bagshaw *et al.*, 1974). Consequently eqn. (1) has been simplified to eqn. (2), which accounts adequately for the results described:



In eqn. (2), E represents frog myosin or its subfragment 1. In addition, experiments are reported in which the rate constants for the ATP-induced dissociation of actin-subfragment-1 complex and the association of actin and subfragment 1 were measured.

Materials and Methods

Proteins

The preparation of myosin and its subfragment 1 and actin from frog leg muscles was carried out as described by Ferenczi *et al.* (1978).

Catalytic-centre activities of myosin are quoted on the assumption that both myosin heads are simultaneously active. Concentrations of myosin and subfragment-1 active sites were calculated from the amount of transient P_i formation when an excess of ATP was mixed with protein (see, e.g., Fig. 1) and multiplying the result by 1.06 to allow for reversibility of the ATP-cleavage step. All proteins were used within 4 days of the frog dissection.

Reagents

Sodium salts of ATP and ADP, and α -chymotrypsin were obtained from Boehringer/Mannheim G.m.b.H., Mannheim, West Germany.

$[^{32}P]P_i$ was obtained from The Radiochemical Centre, Amersham, Bucks., U.K. $[\gamma\text{-}^{32}P]ATP$ was prepared by the method of Glynn & Chappell (1964). ThioITP, listed as 6-mercaptapurine ribonucleoside 5'-triphosphate (tetrasodium salt), was obtained from P-L Biochemicals, Milwaukee, WI, U.S.A. Dithiothreitol was from BDH Biochemicals, Poole, Dorset, U.K., and all other reagents were of analytical grade. Glass-double-distilled water was used throughout.

Quenched-flow measurements

The time course of ATP cleavage by frog myosin and subfragment 1 was measured by two different devices. (1) For reaction times less than 300 ms the device described by Gutfreund (1969) was used. Equal volumes of substrate and enzyme were pushed through a mixing chamber and entered a glass tube into which was inserted a polyethylene tube (1.25 mm internal diam.), which led the reaction mixture into a vial containing 6% $HClO_4$ quenching solution. The final $HClO_4$ concentration was at least 3%. By using a constant flow velocity, the reaction time was varied by altering the length of the tube between 4.5 cm and 130 cm. (2) For reaction times longer than 250 ms a device constructed by Mr. M. A. Geeves and Dr. D. W. Yates (University of Bristol) was used. In this device, a sample of enzyme solution was placed in a vial (17 mm internal diam.) and magnetically stirred. By using an electronic timing circuit that controlled two pneumatically driven syringes, the reaction was initiated by the addition of a sample of substrate from syringe 1 and after a predetermined time interval quenched by the addition of $HClO_4$ from syringe 2 at a final concentration of 3%.

Temperature control was effected either by using the instrument in a cold-room or, for the pneumatic device, by inserting the reaction vial in a water jacket whose temperature was controlled by a Grant Instruments (Cambridge, U.K.) LB8 refrigerated bath.

Samples were subsequently treated as described by Ferenczi *et al.* (1978). In most cases data collection to determine the steady-state rate of ATP hydrolysis in transient experiments (e.g. Fig. 1) was extended beyond the time range shown in the Figures. Typically, five data points were collected during the steady state.

Stopped-flow measurements

The stopped-flow spectrophotometer used was built by Professor H. Gutfreund and Dr. D. W. Yates (University of Bristol). Light provided by a 100W mercury arc lamp was passed through a Farrand Foci grating monochromator and reached the observation cell of the instrument via a quartz light-guide (Schott, Mainz, W. Germany).

Scattered light or fluorescent emission was observed perpendicularly to the incident beam by an EMI 9601 B photomultiplier tube whose signal was filtered by the use of an RC circuit of variable time constant (0–100ms). The signal was amplified before display on a Tektronix 2B67 storage oscilloscope. In the fluorescence mode the emitted light was filtered by UG11 and WG345 filters (Schott). Experiments were performed in a cold-room at 2–5°C. The data were recorded photographically, the negatives enlarged and traced and single exponential rate constants were extracted from the data by the technique described by Gutfreund (1972, p. 118) or Guggenheim (1926).

pH measurements

The pH of the solutions was set at the temperature at which the experiments were conducted by using a Pye-Unicam model 292 Mk.2 pH-meter kept in a cold-room at 5°C, with P_i standards.

Presentation of averaged data

The averaged data are shown as the mean \pm 1 s.e.m. for the numbers of observations (*n*) indicated in parentheses. In the Figures 1 s.e.m. is indicated by vertical bars.

Results

Time course of ATP cleavage catalysed by myosin and its subfragment 1

The time course of Mg²⁺-dependent ATP cleavage by myosin and subfragment 1 was investigated to characterize the steady-state intermediate of the ATPase. When MgATP was mixed with myosin or subfragment 1, its cleavage occurred as a rapid transient phase followed by a much slower steady-state hydrolysis rate.

At 20 μ M-ATP and myosin (16 μ M-heads) (Fig. 1) the observed rate constants of the exponential transient phase and the observed k_{cat} were 0.6 s⁻¹ and 0.003 s⁻¹ respectively. Extrapolation of the steady-state rate to zero time showed that the transient amplitude corresponds to 0.34 mol of P_i liberated/mol of myosin subfragment-1 heads. At 0°C and pH 7.0, in 50 mM-Tris/HCl/0.5 M-KCl/5 mM-MgCl₂ the transient amplitude for P_i liberation averaged 0.31 \pm 0.03 (*n* = 12) mol of P_i/mol of subfragment-1 heads at ATP concentrations at least 20 times the K_m value for ATP (Ferenczi *et al.*, 1978). Towards the end of this work we observed an increase in transient amplitudes that reached 0.52 mol of P_i/mol of subfragment-1 heads at an ATP concentration of 20 μ M. This was probably associated with improved preparative techniques. At ATP concentrations near the K_m (i.e. below 1 μ M) the transient amplitude decreased because the enzyme was not saturated.

In similar experiments, but at lower ionic strength (0.1 M-KCl), subfragment 1 showed analogous behaviour (Fig. 2) and the transient amplitude at high ATP concentrations was 0.30 \pm 0.05 mol of P_i/mol of subfragment-1 heads (*n* = 9).

When the concentration of MgATP was varied under pseudo-first-order conditions, i.e. the ATP concentration was 5–10 times that of the active protein, the transient rate of P_i production increased linearly with the ATP concentration for both myosin and subfragment 1 (Fig. 3). No plateau of the transient hydrolysis rate was observed at ATP con-

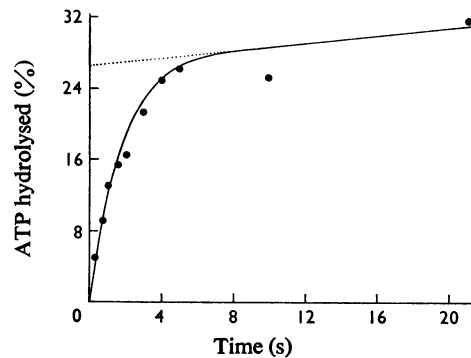


Fig. 1. Time course of ATP disappearance during the reaction of ATP with frog myosin

[γ -³²P]ATP (20 μ M) was mixed with 16 μ M-myosin heads in 0.5 M-KCl/5 mM-MgCl₂/50 mM-Tris/HCl at pH 7.0 and 0–2°C. The rate constant associated with the exponential transient phase is 0.6 s⁻¹ and the amplitude is 0.34 mol of P_i formed/mol of myosin heads. The steady-state rate of P_i formation was measured for 60s. After correction for inactive protein and on the basis that both heads are simultaneously active (i.e. assuming 5.4 μ M active myosin heads), k_{cat} was calculated to be 0.003 s⁻¹.

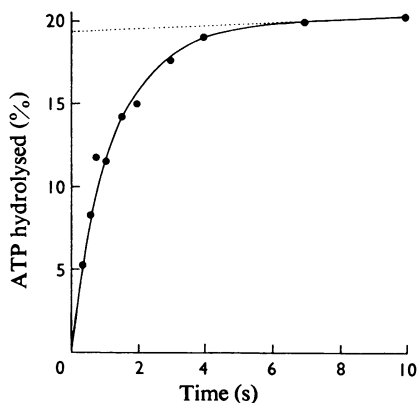


Fig. 2. Time course of ATP disappearance during the reaction of ATP with frog myosin subfragment 1 [γ - ^{32}P]ATP ($10\ \mu\text{M}$) was mixed with $4\ \mu\text{M}$ -subfragment 1 in $0.10\ \text{M-KCl}/5\ \text{mM-MgCl}_2/50\ \text{mM-Tris/HCl}$ at pH 7.0 and 0 – 2°C . The rate constant associated with the exponential transient phase is $0.8\ \text{s}^{-1}$ and the amplitude is $0.48\ \text{mol of P}_i$ formed/mol of subfragment 1. (This amplitude is large compared with the average obtained in these experiments.) The steady-state rate of P_i formation was measured for 60 s. After correction for inactive protein (i.e. assuming $1.9\ \mu\text{M}$ -active subfragment 1) k_{cat} was calculated to be $0.014\ \text{s}^{-1}$.

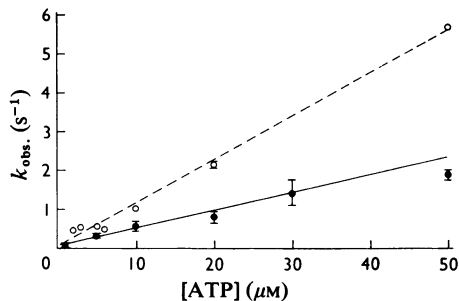


Fig. 3. Observed rate constants, $k_{\text{obs.}}$, associated with the exponential transient phase of P_i formation as a function of ATP concentration

[γ - ^{32}P]ATP in 5–10-fold molar excess over active myosin subfragment-1 heads was mixed with (○) subfragment 1 in $0.10\ \text{M-KCl}$ or (●) myosin in $0.50\ \text{M-KCl}$ in $5\ \text{mM-MgCl}_2/50\ \text{mM-Tris/HCl}$ at pH 7.0 and 0 – 2°C . $k_{\text{obs.}}$ was calculated from data such as that shown in Figs. 1 and 2. Where error bars are not indicated, the data points represent single experiments.

centrations up to $50\ \mu\text{M}$, which shows that the sum ($k'_{+2} + k'_{-2}$) (eqn. 2) is greater than $2\ \text{s}^{-1}$ and $5\ \text{s}^{-1}$ for myosin and subfragment 1 respectively. No ATP concentration higher than $50\ \mu\text{M}$ was used because the amount of subfragment 1 required for

the transient amplitude to be significantly greater than the background [^{32}P]P_i exceeds that obtainable in a single preparation. The second-order rate constant for myosin in $0.5\ \text{M-KCl}$ was $4.3 \times 10^4 \pm 0.4 \times 10^4\ \text{M}^{-1} \cdot \text{s}^{-1}$ ($n = 14$) and for subfragment 1 in $0.1\ \text{M-KCl}$ was $1.1 \times 10^5 \pm 0.2 \times 10^5\ \text{M}^{-1} \cdot \text{s}^{-1}$ ($n = 9$) at pH 7.0, 0 – 2°C .

As indicated in the preceding paper (Ferenczi *et al.*, 1978) the corrected k_{cat} values were $0.004 \pm 0.001\ \text{s}^{-1}$ ($n = 12$) for myosin, expressed per mol of subfragment-1 heads, and 0.011 ± 0.002 ($n = 8$) for subfragment 1 in $0.5\ \text{M-KCl}$ and $0.1\ \text{M-KCl}$ respectively at pH 7 and 0 – 2°C .

Kinetics of ADP dissociation and the influence of P_i

In the previous section it was established that a significant fraction of the Mg^{2+} -dependent ATPase steady-state intermediate is some form of myosin–product complex. Experiments described in this section were performed to measure the rate at which ADP dissociates from the binary myosin–ADP complex (step 4 in eqn. 2). If the rate constant controlling this process were to be much greater than the steady-state rate, the binary complex would not form a significant fraction of the steady-state intermediate of the myosin Mg^{2+} -dependent ATPase.

Two different methods were used. The first consisted of comparing the rate of transient P_i formation after the reaction of ATP with myosin in the presence and absence of ADP. When $20\ \mu\text{M}$ -[γ - ^{32}P]ATP was mixed with myosin that had been incubated with $20\ \mu\text{M}$ -ADP and $100\ \mu\text{M}$ -P_i, the rate of transient P_i formation was $0.28\ \text{s}^{-1}$ compared with $0.96\ \text{s}^{-1}$ in the absence of ADP and P_i (Fig. 4). Further, the transient amplitude in the presence of ADP and P_i was 94% of that in the absence of ADP, which is the same within the experimental error. This result shows that the rate of ADP dissociation is at least $0.28\ \text{s}^{-1}$ and its displacement by ATP was essentially complete. Since k_{cat} for the myosin-catalysed ATP hydrolysis is $0.004\ \text{s}^{-1}$ under these conditions, ADP release from the binary complex is not the rate-limiting step and this binary complex is not a significant fraction of the steady-state intermediate.

In a comparable experiment using $12\ \mu\text{M}$ -[γ - ^{32}P]ATP to displace ADP from subfragment 1 ($2\ \mu\text{M}$ -subfragment 1, $3\ \mu\text{M}$ -ADP, $0.1\ \text{M-KCl}$, $5\ \text{mM-MgCl}_2$, $50\ \text{mM-Tris/HCl}$ at pH 7.0 and 0 – 2°C) the transient P_i amplitude was unaffected by the presence of ADP, but the rate of transient P_i formation decreased from $1.6\ \text{s}^{-1}$ to $0.5\ \text{s}^{-1}$.

The second method was based on the quenching of subfragment-1 fluorescence after the binding of the ATP analogue thioITP in the presence and absence of ADP. Under pseudo-first-order conditions the rate constant of the exponential process

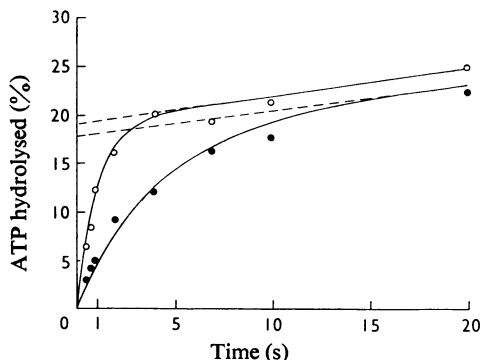


Fig. 4. Effect of ADP on the observed rate constant ($k_{obs.}$) of the exponential transient phase of P_i formation (○), Exponential conditions are as described in Fig. 1. $k_{obs.} = 0.96 s^{-1}$. Corrected $k_{cat.}$ measured over 60s was $0.006 s^{-1}$. ●, Experiment was repeated except that the protein was preincubated with $20 \mu M$ -ADP and $200 \mu M$ - P_i (reaction-chamber concentrations). $k_{obs.} = 0.28 s^{-1}$. Corrected $k_{cat.}$ measured over 60s was $0.006 s^{-1}$.

observed when thioITP reacted with subfragment 1 increased linearly with the thioITP concentration (Figs. 5 and 6). After incubation of subfragment 1 with a 10-fold molar excess of ATP for 45 min to allow complete hydrolysis of the ATP, the resulting complex was allowed to react with $25 \mu M$ - and $100 \mu M$ -thioITP. In each case an exponential process occurred for which the observed rate constants were $0.42 \pm 0.06 s^{-1}$ ($n = 4$) and $0.58 \pm 0.02 s^{-1}$ ($n = 2$) respectively and contrasted with the observed rate constants of $2.3 \pm 0.2 s^{-1}$ ($n = 3$) and $10.6 \pm 3.1 s^{-1}$ ($n = 3$) when the reaction occurred in the absence of ADP. These results support the conclusion from experiments using $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ to displace ADP (Fig. 4), and show that the rate constant of ADP dissociation from the subfragment-1-ADP complex is approx. $0.5 s^{-1}$, which is 50-fold greater than the steady-state rate at pH 7.0 and 0–2°C.

Possible influences of P_i on the product release step(s) of the Mg^{2+} -dependent ATPase were investigated in two ways. First, the addition of P_i to mixtures of ADP and myosin had no effect on the kinetics of ADP dissociation. For example, $100 \mu M$ - P_i was present in the experiment described in Fig. 4, and comparison of this result with experiments performed in the absence of P_i showed that the amplitude and rate constant of the process were not affected. Secondly, up to 50 mM - P_i had no effect on the steady-state rate of 1 mM - $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ hydrolysis catalysed by myosin at pH 7.0 and 0–2°C in the presence of 5 mM - $MgCl_2$ and an ionic strength made up to 0.5 M with KCl.

These results are compatible with P_i release preceding that of ADP during ATPase activity, as is the

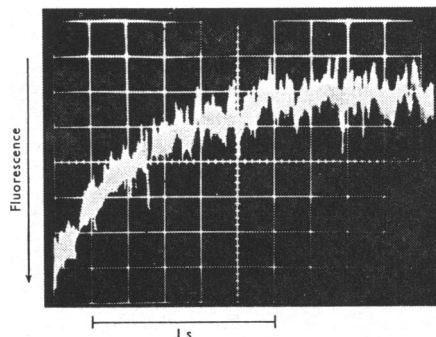


Fig. 5. ThioITP-induced fluorescence change of subfragment 1

ThioITP ($25 \mu M$) was mixed with $1.8 \mu M$ -subfragment 1 in 0.10 M -KCl/ 5 mM - $MgCl_2$ / 1 mM -dithiothreitol/ 50 mM -Tris/HCl at pH 7.0 and 2–5°C. Protein fluorescence was quenched by 10%.

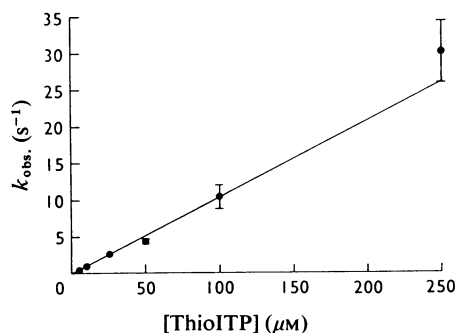


Fig. 6. Observed rate constant ($k_{obs.}$) of the exponential protein-fluorescence quenching during the reaction of thioITP and subfragment 1 as a function of thioITP concentration

Subfragment 1 ($1.8 \mu M$) was mixed with thioITP in 0.10 M -KCl/ 5 mM - $MgCl_2$ / 1 mM -dithiothreitol/ 50 mM -Tris/HCl at pH 7.0 and 2–5°C. The second-order rate constant calculated from the solid line is $1.0 \times 10^5 \pm 0.1 \times 10^5 \text{ M}^{-1} \cdot \text{s}^{-1}$.

likely sequence of events for rabbit myosin (Trentham *et al.*, 1976).

Single-turnover experiments

On the basis of evidence presented in the two previous sections $E \cdot \text{ADP} \cdot P_i$ is essentially the only myosin-products intermediate present in the steady state. In the experiments that follow, the proportion of protein existing as $E \cdot \text{ATP}$ during the steady-state hydrolysis is estimated. Similar experiments have been carried out with subfragment 1 prepared from rabbit

skeletal-muscle myosin by Bagshaw & Trentham (1973)

In these experiments [γ - ^{32}P]ATP was mixed with a molar excess of myosin or subfragment 1, the protein concentration being in large excess of the K_s for ATP. The reaction was allowed to proceed, and at

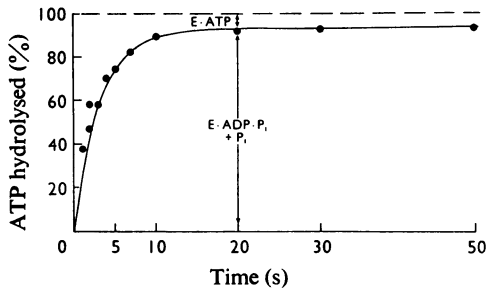


Fig. 7. Kinetics of ATP disappearance during a single-turnover experiment with myosin

[γ - ^{32}P]ATP ($2.5\ \mu\text{M}$) was mixed with $20\ \mu\text{M}$ -myosin heads in $0.50\text{M-KCl}/5\text{mm-MgCl}_2/50\text{mm-Tris}/\text{HCl}$ at pH 7.0 and 0 – 2°C . An experiment was carried out with this preparation of myosin under reaction conditions of Fig. 1. The amplitude of transient P_i formation was $0.40\ \text{mol of P}_i/\text{mol of myosin heads}$. The solid line is a computer simulation of eqn. (2) using a Focal-8 program on a Digital PDP 8 computer. The chosen values for the constants were: $k'_{+1} = 4.8 \times 10^4\ \text{M}^{-1}\cdot\text{s}^{-1}$, $k'_{-1} = 0.004\ \text{s}^{-1}$, $k'_{+2} = 2.0\ \text{s}^{-1}$, $k'_{-2} = 0.14\ \text{s}^{-1}$, $k_{+3} = 0.004\ \text{s}^{-1}$, initial [ATP] = $2.5\ \mu\text{M}$ and initial [active myosin heads] = $8.0\ \mu\text{M}$. The curve is dependent on $K'_2 = k'_{+2}/k'_{-2}$ but is not much affected by the value of k'_{+2} provided $k'_{+2} \gg k'_{+3}$ and $>k'_{+1}$ [active myosin heads]. On this basis, at 20s most of the ATP remaining exists as E·ATP and the hydrolysed ATP is predominantly E·ADP· P_i .

various times the reaction was halted by acid quenching and the fraction of ATP remaining determined. Since protein-bound ATP cleavage is relatively rapid and provided all the ^{32}P is bound at the ATPase active site, the ratio of [^{32}P]P $_i$ /[γ - ^{32}P]ATP present gives, in terms of eqn. (2), the ratio E·ADP· P_i /E·ATP and hence k'_{+2}/k'_{-2} or K'_2 .

One such experiment is shown in Fig. 7 for myosin in 0.5M-KCl . Within 20s 94% of the ATP is cleaved. The remaining ATP is then cleaved over a period of 500s. The rapid phase of ATP cleavage can be analysed as an exponential process with a rate constant of $0.27\ \text{s}^{-1}$ and be correlated with the transient phase of Fig. 1 in that both processes indicate the formation of E·ADP· P_i at a rate controlled by a second-order rate constant of $4.3 \times 10^4\ \text{M}^{-1}\cdot\text{s}^{-1}$ (Table 1). Since myosin is in excess over ATP and the conditions are approximately pseudo-first-order, the observed rate of $0.27\ \text{s}^{-1}$ indicates that the actual active-site concentration of myosin heads is $6.3\ \mu\text{M}$, which compares well with the measured value of $8.0\ \mu\text{M}$ (see the legend to Fig. 7).

From the data of Fig. 3 the rate constant of the process controlling ATP cleavage is at least $2\ \text{s}^{-1}$ so that any protein-bound ATP present at 20s will be in equilibrium, on this time scale, with E·ADP· P_i . If allowance is made for the fact that, as E·ADP· P_i is formed, it will slowly break down, liberating P_i into the medium, the 94%-cleaved ATP at 20s comprises 88% E·ADP· P_i and 6% free P_i plus E·ADP and free ADP. Thus, provided that the ATP present at 20s is bound at the active site, $K'_2 = \text{E}\cdot\text{ADP}\cdot\text{P}_i/\text{E}\cdot\text{ATP} = 15$. Two experiments with myosin gave values for K'_2 of 15 and 14. Experiments with subfragment 1, in 0.1M-KCl , but in otherwise identical conditions, gave a value for K'_2 of 18 (Fig. 8).

In terms of eqn. (2), any E·ATP present at 20s is

Table 1. Comparison of rate constants (eqn. 2) of the Mg^{2+} -dependent subfragment-1 ATPases from frog and rabbit fast-twitch skeletal muscle

	Frog subfragment 1 (0.10M-KCl, pH 7.0, 0–5°C)	Rabbit subfragment 1 (0.050M-KCl, pH 6.9 3°C)*†	Rabbit subfragment 1 (0.10M-KCl, pH 8.0, 21°C)‡§
k'_{+1}	$1.1 \times 10^5\ \text{M}^{-1}\cdot\text{s}^{-1}$	$2.2 \times 10^5\ \text{M}^{-1}\cdot\text{s}^{-1}$	$1.8 \times 10^6\ \text{M}^{-1}\cdot\text{s}^{-1}$
k'_{-1}	$\leq 0.006\ \text{s}^{-1}$	—	$2 \times 10^{-6}\ \text{s}^{-1}$
K'_1	$\geq 1.8 \times 10^7\ \text{M}^{-1}$	—	$7.9 \times 10^{11}\ \text{M}^{-1}$
k_{+2}	$> 5\ \text{s}^{-1}$	6 – $8\ \text{s}^{-1}$	$\geq 160\ \text{s}^{-1}$
K'_2	18	1	9
k_{+3}	$0.011\ \text{s}^{-1}$	$0.036\ \text{s}^{-1}$ (pH 8.4, 0.1 M-KCl)‡	$0.06\ \text{s}^{-1}$
k_{+4}	$0.5\ \text{s}^{-1}$	$0.07\ \text{s}^{-1}$ (pH 8.4, 0.1 M-KCl)‡	$1.4\ \text{s}^{-1}$
k_{cat}	$0.011\ \text{s}^{-1}$	$0.015\ \text{s}^{-1}$	$0.055\ \text{s}^{-1}$
K_m	$0.16\ \mu\text{M}$	$0.071\ \mu\text{M}$ ¶	$0.033\ \mu\text{M}$ ¶

* Sleep & Taylor (1976).

† Taylor (1977).

‡ Bagshaw & Trentham (1974).

§ Mannherz *et al.* (1974).

|| Ferenczi *et al.* (1978).

¶ Calculated from $K_m = k_{\text{cat}}/k'_{+1}$. The validity of this is discussed by Taylor (1977).

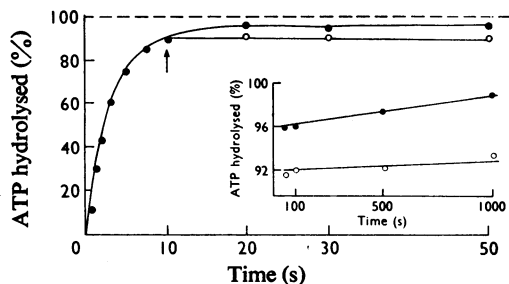


Fig. 8. Kinetics of ATP disappearance during a single-turnover and 'cold-chase' experiment with subfragment 1. ●, [γ - 32 P]ATP (1.0 μ M) was mixed with 9.1 μ M-subfragment 1 in a solution of 0.10M-KCl/50mM-Tris/HCl at pH 7.0 and 0–2°C. ATP decayed exponentially with an observed rate constant of 0.27 s $^{-1}$. ○, At 10s 2mM non-radioactive ATP was added in a 'cold-chase' experiment. An experiment was carried out with this preparation of subfragment 1 under the reaction conditions of Fig. 2. The amplitude of transient P $_i$ formation was 0.33 mol of P $_i$ /mol of subfragment 1. The same simulation as that described in Fig. 7 gave the solid lines with chosen values of: $k'_{+1} = 1.3 \times 10^5 \text{ M}^{-1} \cdot \text{s}^{-1}$, $k'_{-1} = 0.011 \text{ s}^{-1}$, $k'_{+2} = 5.0 \text{ s}^{-1}$, $k'_{-2} = 0.3 \text{ s}^{-1}$, $k'_{+3} = 0.011 \text{ s}^{-1}$, initial [ATP] = 1.0 μ M and initial [active subfragment 1] = 3.0 μ M. The 'cold chase' was simulated by setting $k'_{+1} = 0$ at 10s. Inset: the experimentally observed approach to 100% ATP hydrolysis in the 100–1000s range.

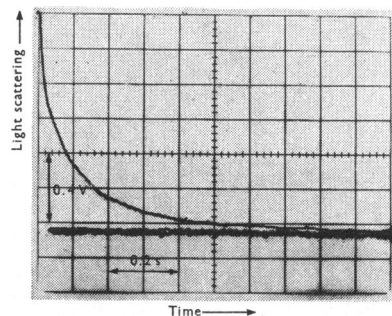


Fig. 9. Frog actin-subfragment-1 dissociation by ATP. ATP (10 μ M) was mixed with 1 μ M frog subfragment 1 and 1.5 μ M frog actin in 0.10M-KCl/5mM-MgCl $_2$ /50mM-Tris/HCl at pH 7.0 and 2–5°C. The observed rate constant of the exponential process was 7.8 s $^{-1}$. The amplitude of the signal decreased slightly as the ATP concentration was increased with a correspondingly larger incursion of the signal into the dead time of the stopped-flow machine.

maintained in equilibrium with E · ADP · P $_i$ and so will decay exponentially over the 20–100s time range with a rate constant equal to k_{cat} . [$= k'_{+2}k'_{+3}/(k'_{+2} + k'_{-2})$]. The inset to Fig. 8 shows that the ATP present at 20s does decay, though with a rate constant less than k_{cat} . However, the small amount of ATP present makes it difficult to measure the rate constant accurately.

'Cold-chase' experiments

A second approach to solving the problem of whether residual [γ - 32 P]ATP represents E · ATP is by a 'cold-chase' experiment. In this case the single-turnover experiment is repeated except that a large excess of non-radioactive ATP is added to the reaction mixture when most or all of the [γ - 32 P]ATP has had time to react with the protein. Subsequently samples of the reaction mixture are quenched into acid in order to follow the time course of [γ - 32 P]ATP hydrolysis. Any [γ - 32 P]ATP not bound at the catalytic site when the non-radioactive ATP is added will be prevented from binding because of the large excess of the latter and so will not be hydrolysed. In addition, however, provided that k'_{-1} is non-zero, any radioactive E · ATP or E · ADP · P $_i$ present when the non-radioactive ATP is added will be capable of

dissociating back into the reaction medium as [γ - 32 P]ATP that will not be hydrolysed.

The final ratio (r) of [γ - 32 P]ATP to [32 P]P $_i$, derived from E · ATP and E · ADP · P $_i$ after a 'cold-chase' experiment has gone to completion, is (Bagshaw & Trentham 1973):

$$r = k'_{-1}k'_{-2}/k'_{+2}k'_{+3} \quad (3)$$

A 'cold-chase' experiment described in Fig. 8 shows that after the addition of non-radioactive ATP at 10s only a relatively small amount of the [γ - 32 P]ATP present at 10s was hydrolysed by 1000s. From the amount of [γ - 32 P]ATP remaining during the 20–50s range in the single-turnover experiment (upper trace in Fig. 8), the maximum [γ - 32 P]ATP that could have been bound at the active site at 10s was 4.5% of the initial ATP, the remaining [γ - 32 P]ATP not having had time to react with the protein. The inset to Fig. 8 shows that the [γ - 32 P]ATP present at 10s decays a further 1.5% in the 50–1000s time range of the 'cold-chase' experiment, although generally the concentration of [γ - 32 P]ATP after a 'cold chase' remained constant. Two possible explanations for this behaviour are either that none of the [γ - 32 P]ATP present at the time of the 'cold chase' was E · ATP or that ATP did dissociate from the active site and $k'_{-1} = 0.006 \text{ s}^{-1}$.

Myosin, in 0.5M-KCl, reacted similarly to subfragment 1 in two 'cold-chase' experiments in that after addition of non-radioactive ATP little or no further hydrolysis of [γ - 32 P]ATP occurred.

Frog actin-myosin interactions

According to the Lymn & Taylor (1971) mechanism, in contracting muscle an intermediate

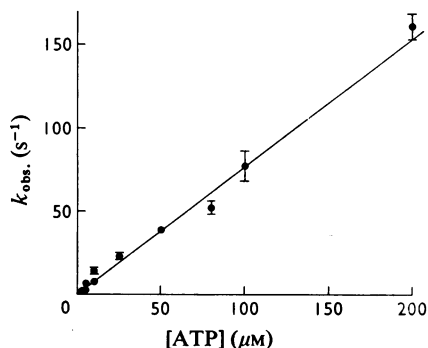


Fig. 10. Observed rate constants ($k_{obs.}$) of actin-subfragment-1 dissociation by ATP as a function of ATP concentration

Reaction conditions were as described in Fig. 9 but with variable [ATP]. The second-order rate constant for the dissociation calculated from the solid line is $7.4 \times 10^5 \pm 0.5 \times 10^5 \text{ M}^{-1} \cdot \text{s}^{-1}$.

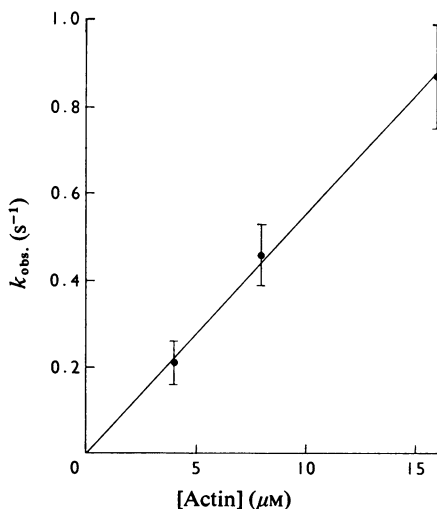


Fig. 11. Observed rate constants ($k_{obs.}$) of the association of actin and subfragment 1 as a function of actin concentration

Actin was mixed with $1 \mu\text{M}$ -subfragment 1 in reaction conditions as described in Fig. 9. The reaction was carried out under pseudo-first-order conditions and $k_{obs.}$ is the rate constant of the exponential process of turbidity increase. From the solid line the second-order association rate constant is $5.5 \times 10^4 \pm 0.8 \times 10^4 \text{ M}^{-1} \cdot \text{s}^{-1}$.

exists that is equivalent to the actin-subfragment-1 complex. For the muscle to shorten at maximum velocities, this complex dissociates rapidly on interaction with ATP.

The dissociation rate constant of the actin-subfragment-1 complex induced by ATP was assessed by measuring the rate of decrease of light-scattering on mixing actin-subfragment 1 with ATP (Finlayson *et al.*, 1969). Fig. 9 shows a typical trace at $10 \mu\text{M}$ -ATP in 0.1 M-KCl , $\text{pH } 7.0$ and $2-5^\circ\text{C}$. The observed rate constant of the exponential process increased linearly with ATP concentration up to $250 \mu\text{M}$ (Fig. 10). The second-order rate constant was $7.4 \times 10^5 \pm 0.5 \times 10^5 \text{ M}^{-1} \cdot \text{s}^{-1}$ ($n = 37$), which suggests that at physiological ATP concentrations (4.5 mM) the dissociation rate constant could be as high as 3400 s^{-1} .

When actin and myosin are mixed together the light-scattering signal increases considerably, indicating that, in the absence of nucleotides, myosin will bind to actin to form an actomyosin complex (Finlayson *et al.*, 1969).

The rate at which subfragment 1 binds to actin was investigated by the same technique as above. The observed exponential rate constant increased linearly with the actin concentration corresponding to a second-order rate constant of $5.5 \times 10^4 \pm 0.8 \times 10^4 \text{ M}^{-1} \cdot \text{s}^{-1}$ ($n = 14$) (Fig. 11).

Discussion

Although in most respects frog myosin can be studied by techniques that have been extensively applied to rabbit myosin, frog and rabbit myosin differ in one major respect that precludes complete duplication of the rabbit myosin studies: namely the lack of any large protein-fluorescence change on ATP or ADP binding. For the rabbit protein such a study of the kinetics of the intrinsic protein-fluorescence change after nucleotide binding enabled K_1 , k_{+2} , k_{-6} and K_7 (eqn. 1) to be determined. Frog subfragment 1 showed a fluorescence change of 2% on ATP binding, which was insufficient to provide adequate signals for binding studies using the stopped-flowspectrofluorimeters available at the time. Nevertheless, the work described above shows that the results obtained from frog myosin and subfragment 1 are compatible with the reaction mechanism proposed for the rabbit myosin Mg^{2+} -dependent ATPase as simplified in eqn. (2).

Although eqn. (2) is the simplest model that accounts for our data, alternatives cannot be rigorously excluded, such as the possibility that the complex formed by the addition of ADP to myosin does not generate E·ADP (i.e. the myosin-ADP complex formed in the forward reaction pathway). For rabbit subfragment 1, however, Bagshaw & Trentham (1974) have shown that the complex formed between the protein and ADP is indeed $\text{M}^* \cdot \text{ADP}$ (eqn. 1), and it therefore seems unlikely that in this respect the frog protein should depart from the general scheme of eqn. (1).

As mentioned in the preceding paper (Ferenczi *et al.*, 1978) one of the major problems arising from the use of frog myosin and subfragment 1 is the high lability of the protein compared with that of the rabbit, resulting in a small proportion of active protein during these experiments. The amplitude of the P_i liberation transients (Figs. 1 and 2) suggests that usually about 30% of the protein was active. This value has been confirmed by titrating subfragment 1 against thioITP. The amplitude of the protein-fluorescence quenching was maximal at about 0.27 mol of thioITP/mol of subfragment 1. It is probable that the protein-fluorescence enhancement for frog subfragment 1 on ATP binding would be much greater if the protein were fully active.

It is likely that our consistent failure to obtain more than 0.5 mol of transient P_i formation/mol of subfragment 1 in quenched-flow experiments is associated with the presence of inactive protein rather than with half-of-the-sites reactivity (Tonomura & Inoue, 1974). The evidence that rabbit subfragment 1 shows full-sites reactivity has been reviewed by Trentham *et al.* (1976) and is further supported by the results of Taylor & Weeds (1977). Taylor (1977) has shown that myosin has similar properties to subfragment 1 with regard to the amount of transient P_i formation.

The results obtained are comparable with previously reported values for rabbit subfragment 1 as shown in Table 1. In Table 1 k'_{+1} has been identified with the second-order rate constant for the binding of ATP to subfragment 1 (Fig. 3). This designation is justified on the basis of Taylor's (1973) arguments, since $k'_{+2} \gg k'_{-1}$. On plotting the observed rate constant for the transient P_i liberation as a function of ATP concentration (Fig. 3), no plateau was observed at high ATP concentrations and hence no value for $(k'_{+2} + k'_{-2})$ can be specified (Taylor, 1973). However, as K'_2 is large, k'_{-2} is only 5–7% of $(k'_{+2} + k'_{-2})$ and hence $k'_{+2} > 5 \text{ s}^{-1}$. This value for k'_{+2} is at least as large as that found for rabbit subfragment 1 at a comparable temperature (Taylor, 1977).

The value of 18 obtained for the cleavage-step equilibrium constant (K'_2) for the frog subfragment-1 ATPase contrasts with the lower value of 1 found by Taylor (1977) and our own unpublished work (M. A. Geeves & D. R. Trentham) for the rabbit subfragment-1 ATPase at 3°C. However, Marston & Tregear (1972) and Marston (1973) have observed that, in relaxed glycerinated rabbit psoas fibres at 1.5°C, at least 90% of the tightly bound nucleotide is present as a myosin-products complex.

The value of k'_{+3} calculated from $k_{\text{cat.}} = k'_{+3}K'_2/(1 + K'_2)$ for frog subfragment 1 is of the same order as values calculated for rabbit subfragment 1 at approximately the same temperature. The rate constant controlling ADP dissociation, k'_{+4} , is larger for frog than for rabbit subfragment 1 at low

temperature. For the frog the large k'_{+4} will result in a low steady-state concentration of $E \cdot \text{ADP}$ (2% of total enzyme at 0°C), whereas for the rabbit at low temperature $E \cdot \text{ADP}$ will be a significant proportion of the steady-state intermediates: 30–40% at 5°C compared with 4% at room temperature (Bagshaw & Trentham, 1974).

Assuming that there is 0.28 μmol of subfragment-1 heads/g of muscle (Ebashi *et al.*, 1969), there will be 0.26 μmol of myosin-bound ADP/g of muscle at 0°C (as $E \cdot \text{ADP} \cdot P_i$ plus $E \cdot \text{ADP}$). According to Table 3 of Barany & Barany (1972), the resting frog semitendinosus muscle contains a total of 0.46 μmol of ADP/g of muscle. After depletion of ATP by iodoacetate treatment of the muscle in the presence of N_2 , rigor complexes form and there remains 0.24 μmol of ADP tightly bound to the protein of muscle; this presumably corresponds to actin-bound ADP. The difference, 0.22 μmol of ADP/g of muscle, agrees well with our calculated value of 0.26 μmol of ADP/g of muscle. These estimates support the conclusion of Marston & Tregear (1972) for rabbit muscle, referred to above, that in muscle the predominant steady-state intermediate is a myosin-products complex, and differs from the interpretation by Barany & Barany (1972) that the main component is a myosin-ATP complex.

Noting that $k'_{+4} \gg k'_{+3}$ and $k'_{+2} \gg k'_{+3}$ and k'_{-1} , the Michaelis-Menten constant K_m for ATP (eqn. 2) is given by:

$$K_m = \frac{k'_{-1} + k'_{+3}K'_2}{k'_{+1}(1 + K'_2)} \quad (4)$$

Substitution of the values given in Table 1 in eqn. (4) gives a K_m of 0.095 μM , which agrees well with our experimental value of $0.16 \pm 0.04 \mu\text{M}$ (Ferenczi *et al.*, 1978). For myosin, substitution of values for the rate constants indicated in the text gives a calculated K_m of 0.12 μM compared with the experimental value of $0.57 \pm 0.15 \mu\text{M}$ (Ferenczi *et al.*, 1978).

The fact that protein-bound ATP is not hydrolysed in 'cold-chase' experiments is in contrast with results obtained with rabbit subfragment 1, but comparable with the results obtained with bovine cardiac-muscle subfragment 1 (Taylor & Weeds, 1976). As already mentioned, two possible explanations are that k'_{-1} is 0.006 s^{-1} or that none of the ATP present at 10s was bound at the active site. The rate of ATP dissociation from rabbit subfragment 1 is $1.0 \times 10^{-7} \text{ s}^{-1}$ (Mannherz *et al.*, 1974; Trentham *et al.*, 1976). The 'cold-chase' experiment therefore suggests there might be an important difference between the frog and rabbit subfragment-1 ATPase mechanisms. However, this type of 'cold-chase' experiment does not provide as valid an approach for determining k'_{-1} as the method of Mannherz *et al.* (1974). In particular the presence of an extraneous species that might promote ATP

dissociation has to be considered, though one obvious candidate, actin, is unlikely to be present in our subfragment-1 preparation. So, until experiments such as those performed by Mannherz *et al.* (1974) are carried out with frog subfragment 1, it is not pertinent to speculate on this apparent difference in the ATPase mechanisms, as its basis may be trivial.

Comparison with physiological data

The steady-state turnover rates of myosin and actomyosin ATPase determined in the study (Ferenczi *et al.*, 1978) may be compared with corresponding data from studies on resting and activated frog muscles. Because of the aggregation of myosin into filaments at low ionic strength which must affect its ability to combine with actin, subfragment-1 data are used for the most part in the comparisons that follow. A resting frog muscle at 0°C consumes 7.3 nmol of O₂/min per g of muscle (Kushmerick & Paul, 1976). Since 6–6.5 mol of ATP is synthesized per mol of O₂, the ATP consumption by resting muscle is approx. 0.75 nmol of ATP/s per g of muscle. Assuming that there are 0.28 μmol of subfragment 1/g of muscle (Ebashi *et al.*, 1969), this corresponds to a turnover rate per subfragment-1 head of 0.0024 s⁻¹. The latter value is only about one-fifth of the $k_{cat.}$ of 0.011 s⁻¹ of the subfragment-1 Mg²⁺-dependent ATPase at 0–2°C, and the discrepancy may be greater, since other ATPases apart from myosin may be active in resting muscle. On the other hand, the $k_{cat.}$ value is 48% of the turnover rate of 0.035 s⁻¹ per subfragment-1 head calculated from the rate of ATP consumption in relaxed skinned fibres at 4°C (Levy *et al.*, 1976). The $k_{cat.}$ value determined for relaxed myofibrils is even higher at 0.05 s⁻¹ (Ferenczi *et al.*, 1978), which may indicate some loss of troponin-tropomyosin control of the actin-myosin interaction.

In maximally working frog muscle, ATP is hydrolysed at a rate of 1.3 μmol/s per g of muscle at 0°C (Kushmerick & Davies, 1969), which gives an ATPase turnover rate of 4.6 s⁻¹ per subfragment-1 head. The corresponding value for an isometric contraction is 1.47 s⁻¹ (Curtin *et al.*, 1974) and a similar value of 0.96 s⁻¹ has been reported for fully activated isometric skinned fibres (Levy *et al.*, 1976). During shortening at the maximum velocity (the state that probably most closely resembles that of actomyosin in solution) the rate is 3.33 s⁻¹ (Kushmerick & Davies, 1969). These values are to be compared with the $k_{cat.}$ of 4.5 s⁻¹ for the actin-activated subfragment-1 ATPase (Table 2 and Ferenczi *et al.*, 1978). Because of the largely unknown effect on the rate constants of the organization of myosin and actin in the myofilament lattice the comparisons cannot be pursued further.

The actin activation of the subfragment-1 ATPase is 440-fold and this compares with a factor of 1400

Table 2. Relation of actomyosin organization and state of the muscle to actomyosin ATPase activity of frog muscle at 0°C and pH7

	$k_{cat.}$ (s ⁻¹)
Actin-activated subfragment 1*	4.5
Actomyosin*	0.11
Myofibrils*	0.5
Isometric muscle†	1.2
Maximally working muscle†	4.4

* Ferenczi *et al.* (1978).

† Mean values taken from references listed in the text.

for the relative turnover rates of muscle shortening at maximum velocity and resting muscle. Considering the diverse approaches used to obtain the various ATP-turnover rates, the degree of consistency in results is satisfactory. A feature of myosin ATPase that distinguishes it from typical enzyme reactions is that it exhibits two extreme activities, one of which depends on the association and dissociation of a second protein during each cycle. Such a high ratio between these two activities is crucial to muscle function and appears difficult to achieve experimentally once the natural environment has been disrupted.

According to the Lymn & Taylor (1971) model the myosin and actomyosin ATPases have a common cleavage step. On this basis the forward rate constant of the cleavage step for the actomyosin ATPase in living muscle corresponds to k'_{+2} . This rate constant (> 5 s⁻¹) is large enough to accommodate the overall rate of hydrolysis observed in maximally working muscle (4.4 s⁻¹).

The observed rate constant of dissociation of frog actin-subfragment 1 at 200 μM-ATP is 150 s⁻¹ (Fig. 10). Our data were too scattered to determine if the observed rate constant showed a plateau at higher ATP concentrations, but only slow or smooth muscles have so far shown saturation rate constants of less than 2000 s⁻¹ (Eccleston *et al.*, 1976; Taylor & Weeds, 1976; White & Taylor, 1976), and accordingly at the physiological ATP concentration of 4.5 mM (Dawson *et al.*, 1977) the dissociation rate is likely to be about 3400 s⁻¹. According to models of contraction such as that of Huxley (1957), the rate of detachment of cross-bridges is rate-limiting for the maximum shortening velocity. A rough calculation of the rate of detachment of cross-bridges required for maximal shortening velocity gives a value about an order of magnitude lower than 3400 s⁻¹ (Simmons & Jewell, 1974). Either the rates of one (or more) of the steps involved in the dissociation of actomyosin are altered in intact muscles by reason of mechanical constraints on the cross-bridges of the type described by Huxley & Simmons (1971) or the step that limits the rate of detachment precedes the binding of ATP.

Our main conclusion from this analysis is that the steady-state and transient kinetic results we have obtained are compatible with the generally accepted hypothesis of how ATPase activities of isolated muscle proteins are related to the resting and active states of muscle.

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