PHOSPHATE BURST IN PERMEABLE MUSCLE FIBERS OF THE RABBIT

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SUMMARY The transient kinetics of ATP hydrolysis in chemically skinned psoas muscle fibers of the rabbit have been measured. Muscles fibers in the rigor state (absence of nucleotide) were relaxed rapidly by the photochemical release of [2-^{3}H]ATP from caged-ATP (P^3-1-(2-nitro)phenylethyl[2-^{3}H]adenosine 5'-triphosphate) in the absence of calcium ions. Rapid freezing of the fiber to stop hydrolysis, followed by analysis of the tritiated nucleotide content allowed the course of the hydrolysis to be determined. The timecourse of ATP hydrolysis was biphasic, with an initial rapid phase occurring at a rate of ~60 s^{-1} at 12°C for fibers exposed to >0.7 mM ATP. The amplitude of the rapid phase was as previously reported (Ferenczi, M. A., E. Homsher, and D. R. Trentham, 1984, *J. Physiol. (Lond.)*., 352:575–599).

INTRODUCTION

In 1954, Weber and Hasselbach reported that the rate of ATP hydrolysis by myosin and actomyosin is greater during the first few seconds of the reaction than later, and also showed that the effect was not due to inhibition by the products of the reaction. This observation was the first step in the study of the mechanism of Mg^{2+}-ATP hydrolysis by muscle myosin. The subsequent experiments of Lymn and Taylor (1970) led to the formulation of a kinetic scheme that described the rapid phase of Mg^{2+}-ATP hydrolysis (Lymn and Taylor, 1971). This scheme accommodated the concepts of independent force generators (A. F. Huxley, 1957) and of cycles of cross-bridge attachment and detachment to the thin filaments (H. E. Huxley, 1969).

Biochemical studies of muscle contraction have relied on solubilized muscle proteins so that the relationship between shortening or tension development and ATP hydrolysis has been lost. Since the basic biochemical problem is to determine how the hydrolysis of ATP to ADP and P_i drives the cross-bridge cycle (Eisenberg and Hill, 1985), it is essential to determine the mechanism of ATP hydrolysis in a system where the ability to generate force is maintained, and also where the three-dimensional organization of the protein filaments is preserved. Attempts at determining biochemical mechanisms in contracting muscle fibers have indeed been carried out, using mechanically or chemically skinned muscle fibers to allow control over the myofilament environment. For example, Cooke and Bialek (1979) and Ferenczi et al. (1984*a*) investigated the dependence of isometric tension and the force-velocity relationship on the concentration of Mg^{2+}-ATP. These studies were limited from the biochemical point of view because it was not possible to change the reaction conditions at a fast enough rate to introduce transient biochemical states in the fibers. This limitation restricted the studies to steady-state biochemical conditions.

The rate at which the concentration of ATP can be changed in a permeable muscle fiber is limited by the rate of diffusion of the nucleotide. For rabbit skeletal muscle fibers that are ~40 μm in diameter, the time taken for the concentration of ATP to equilibrate through the fiber is long compared to the initial rate of hydrolysis by the myosin. The synthesis of P^3-1-(2-nitro)phenylethyladenosine 5'-triphosphate (caged-ATP, Kaplan et al., 1978) has resulted in a technique to overcome this problem. Caged-ATP, which is a biologically inert molecule, diffuses and equilibrates readily across permeabilized muscle fibers. Upon illumination by a pulse of laser-produced ultraviolet light, caged-ATP is photolyzed to release ATP (McCray et al., 1980). The mechanical response of muscle fibers subjected to rapid increases in ATP concentration could thus be studied (Goldman et al., 1982,1984*a*,1984*b*).

Ferenczi et al. (1984*b*) developed the technique further to allow biochemical measurements, as well as measurements of the fiber's mechanical response. The technique requires radioactively-labeled caged-ATP and a rapid freezing apparatus that arrests biochemical processes, thus allowing the measurement of the concentrations of products at intervals during the chemical reaction.

These authors have shown that millimolar concentrations of ATP were released in muscle fibers on a time scale that allowed transient kinetic measurements. The steady-state rate of ATP hydrolysis in a muscle fiber depended on filament overlap and on the presence of calcium ions. The amplitude of the initial rapid phase of ATP hydrolysis was measured and it was found that the number of molecules of ATP hydrolyzed was close to the number of myosin-active sites (cross-bridges) in the muscle fiber. However, the amplitude of the initial phase of ATP hydrolysis varied somewhat with the mechanical state of the muscle: the amplitude was largest for activated muscle fibers (in the presence of calcium ions) and smallest for fibers stretched to no overlap between the thick and thin filaments. The

technique, however, did not allow a measurement of the rate of the initial phase of ATP hydrolysis.

In these experiments the muscle fiber was initially bathed in the experimental solutions, but 300 ms before the laser pulse the fiber was suspended in air. This procedure was necessary to allow rapid freezing of the fiber by the freeze-clamp apparatus. The temperature of the muscle fiber in air was, assumably, close to the temperature of the solution in which the fiber had bathed. I describe here a technique that allows these assumptions to be tested and a modification of the equipment to measure the initial rate of ATP hydrolysis after release of ATP. The rate of the initial phase of ATP hydrolysis was measured for fibers in the absence of calcium ions, after photochemical release of ATP. A rate of $\sim 60\ s^{-1}$ was obtained for fibers at 12°C.

METHODS

The synthesis of tritium-labeled caged-ATP (P^3-1-(2-nitro)phenylethyl [2-^{3}H]adenosine 5'-triphosphate) was as described previously (Ferenczi et al., 1984*b*). Rabbit psoas muscle fibers were chemically skinned and mounted in the apparatus as previously described (Ferenczi et al., 1984*b*). Sandy Lops rabbits weighing <3 kg were used. Experiments were carried out on single fibers, with a cross-sectional diameter of 40 μm. The laboratory air temperature was 22°C and the relative humidity was ~60%.

The apparatus was a development of that used previously at the University of Pennsylvania and also incorporated a ruby laser (Lasermetrics, Inc., Englewood, NJ) that was frequency-doubled with a temperature-phase heated rubidium dihydrogen arsenate crystal (Interactive Radiation, Inc., Northvale, NJ) to provide up to 1 J of energy in a pulse at 347 nm. The primary pulse energy was attenuated by rotation of a stacked-plate polarizer so that muscle fibers were illuminated with 20–40 mJ.

A muscle fiber was held between two hooks, one attached to a motor to change the muscle length, and the other attached to a tension transducer. The fiber was immersed in one of five 12-μl cells that were cut in a stainless steel block mounted on a mechanical stage. The cells could be lowered, leaving the fiber suspended in air. The mechanical stage allowed a different cell to be placed below the fiber, and then raised, thus enabling the solution bathing the fiber to be changed. The muscle fiber was in the light path of the laser beam. At a pre-set time after the pulse of laser light, two copper blocks cooled in liquid nitrogen were released from the liquid nitrogen and brought into contact with each other, trapping the muscle fiber between them. The rapid cooling of the fiber arrested ATP hydrolysis. Extraction of the fiber and analysis of nucleotide content by High Pressure Liquid Chromatography (HPLC) was then carried out (Ferenczi et al., 1984*b*).

Modifications to the Apparatus

The major modification to the apparatus was a change in the laser light path. The laser light path was modified by means of three fused-silica right-angle prisms so that the light illuminated the fiber from above, rather than from the side. The advantage of this arrangement was that the falling copper blocks used for rapid freezing of the fiber did not interrupt the light path during their fall. This meant that there was a much lower limit for the time interval between the pulse of light and the freezing of the muscle fiber: data points were obtained for reaction times as low as 2 ms. The prism closest to the fiber and the cylindrical lens were mounted on rotatable arm so that these elements could be moved away from the apparatus during fiber mounting.

The beam of an He-Ne alignment laser placed on the laser optical bench was directed onto the apparatus through the back reflector of the ruby laser. During alignment, the He-Ne beam illuminated the fiber and facilitated centering of the fiber. An adjustable slit close to the fiber cut off the light falling on the metal hooks, thus minimizing the tension artefact caused by laser light absorbed by the tension transducer hook.

A minor modification was the provision of five interchangeable cells (as opposed to two in the previous design) in which the muscle fiber could be immersed. The additional cells accelerated the protocol by reducing the number of cell rinses.

Protocol

A 6-mm long segment of single muscle fiber or of a small bundle of up to three fibers was mounted in the apparatus and bathed in experimental solutions as described previously (Ferenczi et al., 1984*b*). The physiological response of the fiber was evaluated by recording the tension development after immersion in activating solution (5 mM Mg^{2+}-ATP, 2×10^{-5} M free Ca^{2+}, pH 7.1, ionic strength = 200 mM) for 2–10 s before returning it to relaxing solution (5 mM Mg^{2+}-ATP, $<1 \times 10^{-8}$ M free Ca^{2+}). The fiber cross-section and sarcomere length were measured and the fiber was transferred to rigor solution (0 Mg^{2+}-ATP, $<1 \times 10^{-8}$ M free Ca^{2+}) in which rigor tension developed. The fiber was then transferred to a solution containing: 1.2 mM $MgCl_2$ (1 mM free Mg^{2+}), 13 mM EGTA (ethyleneglycol-bis-[amino-ethyl ether] tetraacetate, resulting in $<1 \times 10^{-8}$ M Ca^{2+}), 30 mM HDTA (diaminohexane tetraacetate), 10 mM glutathione, 2–5 mM caged-[^{3}H]ATP, 100 mM TES (*N*-tris[hydroxymethyl]methyl-2-aminoethanesulphonate). After a 2-min equilibration period the cell in which the fiber bathed was lowered and 300 ms later a pulse of laser light illuminated the fiber, releasing 0.7–2.0 mM [2-^{3}H]ATP and causing relaxation of the fiber. The fiber was then rapidly frozen 2–300 ms after the pulse of light. Nucleotide content was analyzed by HPLC as previously reported. Fibers frozen without illumination by laser light allow for the calculation of background radioactivity, namely the amount of [2-^{3}H]ADP, [2-^{3}H]ATP and any other tritiated material in the caged-ATP stock. These fibers also provide a measure of the amount of photolysis and hydrolysis that occurs during the work-up of the samples. Typically these control fibers showed the following distribution of radioactivity: caged-ATP 98.0%, ATP 1.3%, ADP 0.5%, AMP 0.2%. The contaminant levels of tritiated nucleotides in the [^{3}H]caged-ATP stock was only marginally lower than these figures. The stock solution of nonradioactive caged-ATP solutions into which the tritiated caged-ATP was added before experiments was >99% pure.

The experimental solutions, fiber freezing technique, nucleotide extraction, and tritiated nucleotide analysis have been described previously (Ferenczi et al., 1984*b*). Where the mean and error values are given, the error is one standard error of the mean.

In activating solution, at bath temperatures of 12.2–14.2°C (median of 13.4°C) and 9.7–10.6°C (median of 10.0°C), isometric tensions were $140 \pm 5\ kN \cdot m^{-2}$ ($n = 39$) and $116 \pm 10\ kN \cdot m^{-2}$ ($n = 21$), respectively. For comparison Dantzig and Goldman (1985) obtained a value of 161 $kN \cdot m^{-2}$ at 20°C for an identical rabbit psoas fiber preparation.

The fiber cross-sectional area was $3{,}400 \pm 130\ \mu m^2$ ($n = 44$) and the sarcomere length was on average 2.7 μm, corresponding to ~90% of maximal overlap of the filaments for rabbit skeletal muscle. The fiber cross-sectional area was much enlarged by the skinning process (Matsubara and Elliott, 1972). The fiber volume was either calculated by multiplying the fiber cross-sectional area (measured optically in the center of the fibre, Ferenczi et al., 1984*b*) by the length of the copper blocks in which the fiber segment was frozen, or by the amount of tritium extracted from the fiber compared to the tritium concentration in the bathing solution. The ratio of the volume measured optically to that calculated from the total radioactivity in the fiber was 1.09 ± 0.05 ($n = 34$). Since >95% of the radioactivity was extracted from the fiber, as shown by measurement of the residual radioactivity in the fiber after extraction, this ratio shows that there was no appreciable volume of water that accompanied the fiber out of the experimental cell as an aqueous layer, and that surface tension does not draw water out of the fiber as it passes through the air/water interface. In a few instances, the ratio was

greater than 2, indicating that considerable fiber material was lost during the extraction procedure. In these cases the data were rejected.

RESULTS

Fiber Temperature

In the experiments of Ferenczi et al. (1984*b*) as well as in the experiments described here, the muscle fiber is immersed in caged-ATP solution for a few minutes in a cell at a known temperature, but 300 ms before the laser pulse, the cell is removed so that at the time of the laser pulse the fiber is suspended in air and remains in air until it is trapped by the freezing copper blocks. As there was no available technique to measure the actual fiber temperature at the time of the laser pulse, it had been assumed previously that the fiber temperature during the critical experimental period was close to the temperature of the liquid in the cell in which the fiber had been immersed.

Microscopical observation of the fiber diameter after suspension of the fiber in air shows rapid shrinkage due to evaporation of the fiber water (Fig. 1). Fibers shrink to ~15% of their volume with a half-time of ~10 s and the fibers swell back to their original volume after reimmersion in the bathing liquid. Although beading of water on the fiber surface is sometimes observed, shrinkage is not due to accumulation of water into beads as beads shrink at approximately the same rate as the other parts of the fiber. Detergent in the bathing liquid reduces the beading effect but does not alter the shrinkage rate. Pre-incubation of the fiber in silicone oil (viscosity of 10 cs) does not reduce the rate of water loss, presumably because the oil fails to form an impermeable layer around the fiber.

Evaporation of the fiber water results in cooling. Assuming that the heat of evaporation is provided solely by the heat content of the fiber, the initial rate of cooling can be calculated to be of the order of 40°/s. Cooling occurs until the fiber temperature approaches that of the dew point. The evaporation rate of the fiber gradually decreases, during which time the fiber temperature remains approximately constant because the heat provided by the surrounding air compensates that lost through evaporation.

Experiments in which a wet copper-constantan thermocouple (1.5 mg when dry) replaces the fiber show that cooling from 23° to 13°C is achieved in ~3 s with an initial rate of cooling of 15°/s. The thermocouple data cannot be used to calculate the muscle fiber temperature because it is difficult to estimate the amount of water wetting the thermocouple, and therefore the heat capacity of the wet thermocouple. The total heat capacity of a muscle fiber is, however, at least an order of magnitude less than that of the wet thermocouple so that the fiber cooling rate is considerably faster.

These considerations suggest that the fiber reaches a temperature close to the dew point temperature in a fraction of a second. The realization that the temperature of a fiber in air is close to the dew point, rather than at the temperature of the liquid in which the fiber had been bathing, leads to an explanation for the changes observed in the isometric tension as the fiber is moved from the

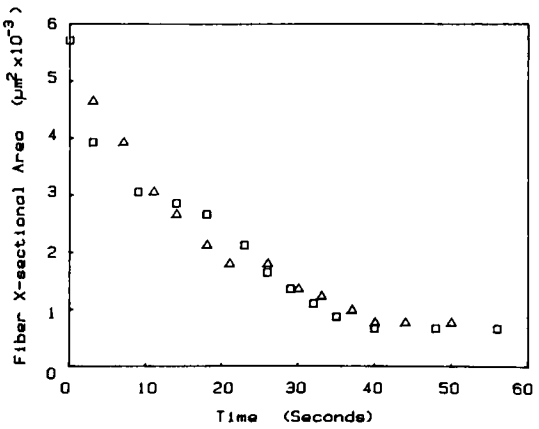

FIGURE 1 Fiber shrinkage in air. Measurements of the fiber diameter through a 40× Zeiss objective (Carl Zeiss Inc., Thornwood, NY) and a 25× Periplan Leitz eyepiece (E. Leitz Inc., Rockleigh, NJ) were made before, and as soon as possible after, removal of the bathing liquid. The fiber cross-section was assumably circular for these measurements. Measurements were carried out twice on the same fiber, at the same position along the length of the fiber. Before and between the measurements, the fiber was immersed in relaxing solution for ~10 min. Squares and triangles indicate the first and second measurements, respectively. A semilogarithmic plot linearized the data satisfactorily, giving a half-time for shrinkage of 9 s for both experiments. The bath temperature was 20°C.

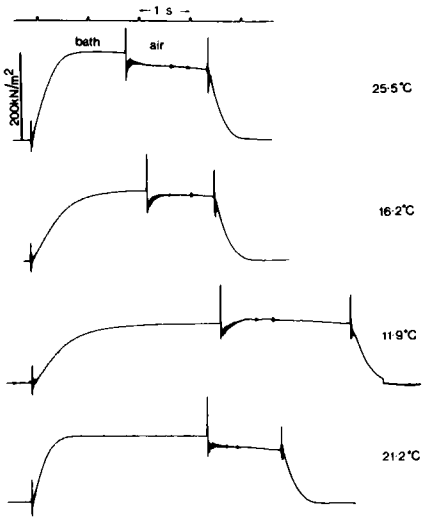

FIGURE 2 Fiber tension in air. Four consecutive isometric contractions of a single rabbit muscle fiber are shown, following the protocol described in the text. The bath temperature was varied in the course of the experiment. At the end of the experiment, isometric tension was 74% of the tension at the beginning of the experiment in a bath at 21°C. The cross-sectional area of the fiber was 3,900 μm^2, as determined from fiber width and thickness measurements (Blinks, 1965) and its sarcomere length was 2.7 μm. No adjustments were made to the composition of the bathing solution to account for the change in temperature. Solution composition was calculated at 20°C.

bathing liquid to air. Such a tension change is shown in Fig. 2 and can be seen in Fig. 3 *A* of Ferenczi et al. (1984*b*). In Fig. 2 a muscle fiber is incubated in relaxing solution ($<10^{-8}$ M free Ca^{2+}, calculated at 20°C) at the temperature shown. The fiber is then transferred to activating solution (2×10^{-5} M free Ca^{2+}, calculated at 20°C) at the same temperature, and tension rises. During the transfer, the fiber is momentarily suspended in air. When the isometric tension plateau is reached, the fiber is removed from the activating solution bath and transferred to a relaxing solution bath. When the fiber is removed from the activating solution bath, isometric tension rapidly readjusts to a new level. The isometric tension in the bath as well as the rate of tension rise are dependent on the bath temperature. The tension level in air is, however, independent of the bath temperature. At a bath temperature of 11.9°C, the tension in the bath is very nearly equal to the tension in air.

Surface tension of the fiber water causes some tension to be recorded by the transducer when the fiber is suspended in air. This tension is measured by comparing the transducer signal for a relaxed fiber in air and immersed in the relaxing solution. At these low tension levels, however, the surface tension on the tension transducer hook itself causes some variability in the signal when the hook is immersed in liquid. The surface tension on the hook is measured independently by removing the fiber from the apparatus. On average, the surface tension effect on the fiber suspended in air is ~10% of the tension changes observed here as a function of temperature. No corrections are made for surface tension.

The ratio of isometric tension in air and in the bath obtained for the fibers used in the experiments described below is shown in Fig. 3. In most experiments, the bath temperature is either set at ~13°C or ~10°C. The isometric tension in air shows that the fiber temperature at the time of the experiments is on average 12°C, which is close to the dew point. The dew point was, however, only determined to ±1°C. The relationship between the ratio of tensions and temperature was not linear over a 10–25°C range, being lower for temperatures above 15°C.

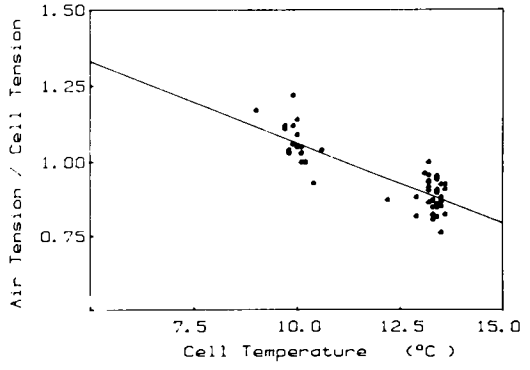

FIGURE 3 Ratio of fiber tension in air and in solution, for the fibers used in the freeze-clamping experiments. The cell temperature was set at around 13° or 10°C. Tension in air was close to the tension in the cell.

Rapid Phase of ATP Hydrolysis

Upon liberation of [2-^{3}H]ATP by the laser pulse, fibers that develop rigor tension relax over a period of 200 ms or so with an approximate rate of 11 s^{-1} (Ferenczi et al., 1984*b*; Goldman et al., 1982, 1984*a*). The relaxation profile is complex, with an initial rise of tension followed by relaxation to the resting level.

At various intervals following the pulse of light the fibers are frozen by freeze-clamping between copper blocks cooled to liquid-nitrogen temperature. After extraction of the nucleotide and analysis by HPLC, the concentration of ADP in the fiber is calculated. The amount of ATP released by the pulse of laser light is also determined, and is found to vary between fibers because of difficulty in maintaining the beam focused consistently on the fibers. The amount of ATP released by a single pulse varies between 15% and 70% of the caged-ATP. The results for fibers in which the laser pulse liberated 0.7–1.6 mM ATP are pooled as, within the precision of the method, it appears that the rate of hydrolysis remains constant in this range of substrate concentration. Below 0.7 mM ATP, the rate of hydrolysis varies as a function of ATP concentration, indicating that saturation is not reached.

The time interval between the pulse of light and freezing by the copper blocks is established by measuring electrical contact between the blocks, and in the present series of experiments is kept below 300 ms. The time between the trigger to the solenoid that holds the copper blocks in the liquid nitrogen cups and the freezing of the fiber varies by ~30 ms between trials because of the varying weight of liquid nitrogen in the cups that altered the inertia of the freeze-clamping apparatus. This uncertainty means that, although the actual reaction time in each trial is accurately measured, the experimental data cannot be collected at predetermined times. To see the reaction profile clearly, averaging of the data is necessary but because of the timing uncertainty described above the data points are grouped over small ranges of reaction times.

In these experiments, chemical data are obtained during the relaxation phase: measurements of ADP formation are carried out as the tension approaches the resting level. The data shown in Fig. 4 indicate that under our experimental conditions the observed rate for the initial phase of ATP hydrolysis is ~60 s^{-1}, and that the concentrations of ADP formed by the end of this phase is 160 ± 17 μM.

INTERPRETATION OF THE RESULTS

The data of Fig. 4 are adequately described by a single exponential with a rate constant of 60 s^{-1}. However, to assign a value for the ATP hydrolysis step of the actomyosin ATPase it is necessary to take into account the overall

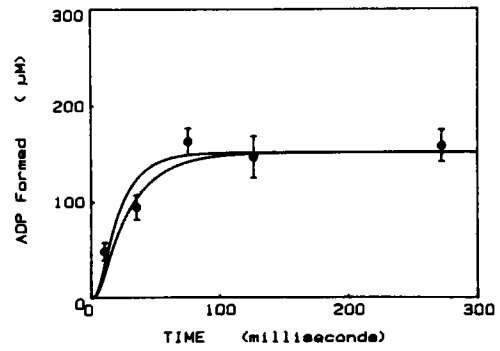

FIGURE 4 The initial phase of ATP hydrolysis. The circles are averages for fibers pooled in time bins: 0–20 ms (average time 10.3 ms, $n = 11$). 21–50 ms (average time 35.3 ms, $n = 11$), 51–90 ms (average time 75.5 ms, $n = 4$) 91–200 ms (average time 126.6 ms, $n = 8$) and 201–300 ms (average time 272.2 ms, $n = 6$). Error bars are plus or minus one standard error of the mean. The *arrow* shows the amplitude of the rapid phase of ATP hydrolysis obtained by Ferenczi et al. (1984*b*) and corresponds to the end-point of the reaction observed here. The *lower* curve is obtained for a simulation of Scheme I with $k_{-1} = 0\ s^{-1}$, $k_{+2} = 40\ s^{-1}$ and the *upper* curve with $k_{-1} \cdot [A] = 10\ s^{-1}$ and $k_{+2} = 60\ s^{-1}$. The other parameters are identical for both curves: $k_{phot} = 58\ s^{-1}$, $k_{+1} = 1 \times 10^{+6}\ M^{-1} \cdot s^{-1}$, $k_{-2} = 0\ s^{-1}$, $k_{+3} = 0.01\ s^{-1}$, the nucleotide concentration is 1 mM and the active site concentration is 152 μM. The curves were insensitive to the value of k_{-1} for $k_{-1} \cdot [A] < 200\ s^{-1}$.

kinetic scheme for the hydrolysis of ATP, and also the kinetics of the release of ATP from caged-ATP.

The kinetic scheme below (Scheme I) is sufficient to explain the experimental data

$$\text{caged-ATP} + \text{light} \xrightarrow{k_{phot}} \text{ATP}$$

$$A \cdot M + \text{ATP} \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} A$$

$$+ M \cdot \text{ATP} \underset{k_{-2}}{\overset{k_{+2}}{\rightleftharpoons}} M \cdot \text{ADP} \cdot P_i \xrightarrow{k_{+3}} M + \text{Products}$$

Scheme I

M represents myosin heads and A represents actin. The reaction scheme describes dissociation of actomyosin by ATP before hydrolysis, and no reattachment of the cross-bridges is assumed.

The behavior of the reaction scheme was simulated on a computer by using values for the rate constants obtained from the literature and from our work in an attempt to obtain limits for the value of k_{+2}, the rate constant for the hydrolysis step.

The Value of k_{phot}

The photochemical release of ATP from caged-ATP is limited by a dark reaction that has a Q_{10} of 2.2 and a rate of 118 s^{-1} at 20°C in the medium of the experiments described here (J. A. Mc Cray, H. Gutfreund, and D. R. Trentham, personal communication). At a temperature of 12°C, the rate of release of ATP from caged-ATP (k_{phot}) is therefore 58 s^{-1}. The rate of ATP release from caged-ATP by the dark reaction of the photochemical process has a relatively small influence on the observed rate of ADP production in the experiment provided the total amount of ATP produced is large compared to the myosin active site concentration. In the experiments here the ATP concentration is >0.7 mM.

The effect of the dark reaction for the release of ATP from caged-ATP can be seen as the initial lag in the simulated curves of Fig. 4.

Computer Simulations

In simulations of Scheme I with $k_{+1} = 1 \times 10^6\ M^{-1} \cdot s^{-1}$, $k_{-1} \cdot [A] \leq 10\ s^{-1}$, $k_{-2} = 0\ s^{-1}$, values for k_{+2} of ~40–60 s^{-1} are obtained (Fig. 4). The observed rate constant is $k_{+2} + k_{-2}$, so that 40–60 s^{-1} is the value for k_{+2} if $k_{-2} = 0$. Although thre is no direct measurement of k_{-1}, the data in the literature (e.g., Sleep and Hutton, 1978) indicate that k_{-1} is small. The rate-limiting step of the ATPase in the absence of calcium ions controlled by k_{+3}, is slow with $k_{+3} \leq 0.1\ s^{-1}$ (Ferenczi et al., 1984*b*).

The Value of K_2

For [ATP] > $[M_0]$ the amplitude of the initial phase of ATP hydrolysis (extrapolated to zero time) is given by

$$[M \cdot \text{ADP} \cdot P_i] = \frac{[M_0]}{1 + 1/K_2},$$

where $[M_0]$ is the total myosin head concentration in the fiber.

With large K_2, the amount of ADP formed during the transient phase is close to the active site concentration. The amount of ADP formed in the rapid phase of the reaction measured by Ferenczi et al. (1984*b*) was close to their determination of the active site concentration (152 μM) and the error on the data indicates that K_2 is unlikely to be <7. Other workers (e.g., Marston and Tregear, 1972) calculated a myosin active site concentration in muscle fibers of ~200 μM. With this value the size of the burst in these experiments indicates a value of K_2 of 3. With this value and with $k_{+2} + k_{-2} > 40\ s^{-1}$, the lower limit for k_{+2} is 30 s^{-1}. The value for K_2 obtained for acto-subfragment 1 in solution (e.g., Rosenfeld and Taylor, 1984; Stein et al., 1984; Biosca et al., 1984) might be somewhat lower than K_2 in fibers although the different conditions of temperature and ionic strength could account for the difference.

The Relaxation Phase is Slower than the Rapid Phase of ATP Hydrolysis

As cross-bridge detachment in the absence of calcium ions results in relaxation, it would be expected that relaxation is faster than ATP hydrolysis. This, however, is not observed. The time course of tension relaxation is complex and

cannot be fitted by a single exponential (Goldman et al., 1984*a*). The measured half-time for relaxation gives a rate of 11 s^{-1} for the overall relaxation process, (Ferenczi et al., 1984*b*) as compared to 60 s^{-1} for hydrolysis. Goldman et al. (1984*a*) suggested that the cooperative mechanism for activation of the thin filament by rigor cross-bridges (Bremel and Weber, 1972) accounts for the transient tension rise before final relaxation, and thus to the slow overall relaxation. According to this mechanism, detached cross-bridges reattach to the thin filament, even in the absence of calcium ions, so long as there is a significant proportion of attached cross-bridges.

Hydrolysis of ATP by Attached Cross-Bridges

Scheme I shows hydrolysis occurring for detached cross-bridges only. It is known, however, that in solution at least, hydrolysis by attached cross-bridges is possible (Stein et al., 1979, 1985) and the data presented here does not conflict with this. By this hypothesis $A \cdot M \cdot$ ATP and $M \cdot$ ATP are in rapid equilibrium. k_{+2} then represents the average of the rate constants of hydrolysis of $M \cdot$ ATP and of $A \cdot M \cdot$ ATP weighted by the relative proportion of each.

The Large Amplitude of the Transient Phase

The data of Ferenczi et al. (1984*b*) suggest that the initial amount of hydrolysis may be >1 mol ATP/mol active site in the presence of calcium ions. Such a large amplitude for the transient phase of ATP hydrolysis would indicate a qualitative difference between ATP hydrolysis in fibers and for the isolated proteins. One possible explanation is that the observed rapid phase is not a single process, but consists of a burst followed by a period during which cross-bridges cycle fast (with a high rate of ATP hydrolysis), release of hydrolysis products, and rebinding of ATP. Such a rapid phase of hydrolysis would be expected for shortening fibers. Homsher et al. (1984) have shown that the ATP hydrolysis rate for a frog muscle shortening at one-half the maximal shortening velocity is five times the rate observed for a muscle contracting isometrically. Higher amounts of hydrolysis during the transient phase were observed by Ferenczi et al. (1984*b*) for experiments carried out in the presence of calcium ions at full filament overlap, where there were lower amounts of hydrolysis when the fibers were stretched to a length preventing the interaction of myosin and actin. This is consistent with the idea that some of the initial hydrolysis of ATP is caused by a transient period during which internal shortening occurs. This would result in a somewhat lower estimate for K_2 than calculated above. If P_i release precedes the rate-limiting step (Hibberd et al., 1985*b*; Hibberd and Trentham, 1986), Scheme I is an oversimplification and again the true value for K_2 may be lower than is suggested at first sight by the data.

Two-Headed Myosin

In the experiments described above the muscle fiber is initially in rigor, a state in which both heads of the myosin are bound to actin, and neither head has any nucleotide bound to it. This muscle state is not physiological, as it is only in rigor mortis that total nucleotide depletion occurs. In the course of ATP hydrolysis a myosin head will be transiently without bound nucleotide, but the lifetime of such a state is very short at physiological ATP concentrations. It is therefore very unlikely that both heads of a myosin molecule would be concomitantly without bound nucleotide. The question of the validity of these experiments for the study of muscle contraction thus arises if the starting material is not found under physiological conditions. For example, a cooperative interaction between the two heads might take place in the absence of nucleotide to induce a myosin state in which one of the myosin heads behaves in a manner not found under physiological conditions.

Marston (1973) showed that in the steady-state, muscle fibers contain bound ADP. The steady-state ^{18}O-exchange studies of Hibberd et al. (1985) have shown that the hydrolysis step in muscle fibers is reversible. In these two respects at least, the results described here for transient experiments that start from an initial nonphysiological, rigor state of the muscle fiber reveal kinetic processes comparable to those observed in steady-state studies.

Hydrolysis in Fibers and in Solution

Goldman et al. (1984*b*) obtained a value of 80–100 s^{-1} for the rate of reformation of force in rabbit fibers at 20°C. As discussed by Hibberd and Trentham (1986) this rate might be that of the hydrolysis step or of a step subsequent to it. The value of 40–60 s^{-1} at 12°C for the hydrolysis rate constant suggests that the rate of force generation might be limited, at least partly, by the rate of hydrolysis.

Conclusion

The temperature of muscle fibers suspended in air has been determined by a technique that uses the fiber isometric tension as a temperature-sensitive probe.

k_{+2} in muscle fibers at 12°C and pH 7.1 is in the range 40–60 s^{-1}, provided that $k_{-1} \leqq 10$ s^{-1} and, as the data suggest the binding of ATP does not limit the hydrolysis step. This is similar to the kinetics of ATP cleavage for actomyosin in solution though the equilibrium constant for the reaction at low ionic strength appears to be smaller.

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