Nucleotide Turnover Rate Measured in Fully Relaxed Rabbit Skeletal Muscle Myofibrils

KATHRYN H. MYBURGH, KATHLEEN FRANKS-SKIBA, and ROGER COOKE

From the Department of Biochemistry and Biophysics and the Cardiovascular Research Institute, University of California at San Francisco, San Francisco, California 94143

ABSTRACT Steady state measurements of the ATP turnover rate of myosin cross-bridges in relaxed living mammalian muscle or in in vitro systems are complicated by other more rapid ATPase activities. To surmount these problems we have developed a technique to measure the nucleotide turnover rate of fully relaxed myosin heads in myofibrils using a fluorescent analogue of ATP (mant-ATP). Rabbit myofibrils, relaxed in 1.6 mM ATP, were rapidly mixed with an equal volume of solution containing 80 μM mant-ATP and injected into a fluorimeter. As bound ADP is released, a fraction of the myosin active sites bind mant-ATP and fluorescence emission rises exponentially, defining a rate of nucleotide turnover of 0.03 ± 0.001 s^{-1} at 25°C ($n = 17$). This rate was approximately equal to one half that of purified myosin. The turnover rates for myosin and myofibrils increased between 5° and 42°C, reaching 0.16 ± 0.04 s^{-1} and 0.06 ± 0.005 s^{-1}, respectively, at 39°C, the body temperature of the rabbit. If the rate observed for purified myosin occurred in vivo, it would generate more heat than is observed for resting living muscle. When myosin is incorporated into the myofilament lattice, its ATPase activity is inhibited, providing at least a partial explanation for the low rate of heat production by living resting muscle.

INTRODUCTION

The rate of ATP utilization of resting muscle has been determined in a variety of experimental systems from living muscle to purified proteins, with widely varying results. For instance, in relaxed living frog muscle, an upper limit on the myosin ATP turnover rate has been estimated from measurements of oxygen consumption (Kushmerick and Paul, 1976). Assuming that all the oxygen consumed was used to resynthesize the ATP used by myosin, the myosin turnover rate per head would be very slow: of the order of 0.002 s^{-1} at 0°C. Since this value includes ATP utilization by enzymes other than myosin, the actual myosin ATPase rate in intact muscle is most likely even lower. In contrast, at a similar temperature the ATPase rate of pu-

Address correspondence to Roger Cooke, Department of Biochemistry and Biophysics, University of California at San Francisco, San Francisco, CA 94143.

K. Myburgh's current address is MRC/UCT Bioenergetics of Exercise Research Unit, University of Cape Town Medical School, Observatory, 7925, South Africa.

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rified myosin is 0.011 s^{-1} per myosin head, approximately five times faster than the maximal rate obtained from the data from living fibers (Ferenczi, Homsher, Simmons, and Trentham, 1978*b*). This comparison shows that a mechanism must exist that further inhibits the myosin ATPase activity in the living frog fiber; however, this mechanism remains unexplained. To determine whether a similar inhibition of the myosin ATPase occurs in mammalian fibers, we have developed methods to measure the myosin enzymatic rate in a relaxed in vitro system.

Measurement of the actual turnover rate of myosin in an in vitro system presents several technical difficulties. The ATPase activity of myosin mixed with reconstituted thin filaments is much greater than the rate inferred from living muscle because of the difficulty in completely reconstituting the regulatory proteins (for a review see Adelstein and Eisenberg, 1980). Myofibrils represent a preparation with filament arrays that are similar to those of intact fibers, but in which kinetic measurements in suspension are still possible. However, the ATPase rate of relaxed frog myofibrils, 0.05 s^{-1} per myosin head, is faster than that of living fibers or of purified myosin (Ferenczi, Homsher, Trentham, and Weeds, 1978*a*). This high rate of ATPase activity may come from other enzymes that are in the preparation, or it may be a result of insufficient inhibition of the actomyosin interaction by regulatory proteins that have been damaged during the preparation of the myofibrils. If such damage occurred, some portions of the myofibrils would be unable to relax and would therefore use ATP at the same rate as active myofibrils. Because the rate of active cross-bridges is much faster than that of the fully relaxed cross-bridges, a very small fraction of active cross-bridges, $<1\%$, can dominate the ATPase activity of the preparation. We hypothesize that in steady state measurements, the high ATPase rate of proteins other than myosin, or of damaged myofibrils, masks the true ATPase rate of those myofibrils which are fully relaxed and hydrolyzing ATP slowly, at a rate similar to that inferred from living muscle.

In this paper we describe a method that allows the determination of the nucleotide turnover rate of the fully relaxed myosin head despite the presence of other more rapid ATPase activities resulting from damaged regulatory proteins or from other enzymes. The turnover rate of the myosin in truly relaxed skinned skeletal myofibrils was measured by rapidly introducing a fluorescent ATP analogue, 2′(3′)-*O*-(*N*-methylanthranoloyl)-ATP (mant-ATP) into a myofibril or myosin preparation that is relaxed in ATP. As the ATP is turned over, the mant-ATP replaces the nonfluorescent nucleotide on a portion of the myosin heads. This process has a time course that is slow, approximately equal to the true turnover rate of nucleotides bound to truly relaxed myosin heads. The more rapid turnover by active heads in the preparation or by other enzymes reaches a steady state quickly, before data collection on the time scale of our experiments begins. Thus, the fraction of fluorescent nucleotides bound to these active sites does not change during the measurement of the slower turnover rate observed for myosin, and the presence of these faster processes poses little problem. Mant-ATP has been shown to increase its fluorescence emission by ~2.5-fold on binding to myosin (Hiratsuka, 1983; Cremo, Neuron, and Yount, 1990; Woodward, Eccleston, and Geeves, 1991). Mant-ATP is a good substrate in myosin and actomyosin preparations with rates for binding, hydrolysis and steady-state turnover that are not too different from those in ATP

(Woodward et al, 1991). Furthermore, Ferenczi, Woodward, and Eccleston (1989) have shown that skinned rabbit fibers contract and relax well with mant-ATP as substrate. Therefore, mant-ATP is an excellent analogue for measuring nucleotide turnover rates and its binding to myosin in myofibrils should provide an accurate method for monitoring the myosin ATPase rate.

We found that the nucleotide turnover rate of fully relaxed myofibrils, measured using the fluorescent nucleotide, is slower than that determined by steady state methods, showing that the conventional steady state activity observed in myofibrils is not a good measure of the true myosin ATPase activity. In addition, the rate for myosin in myofibrils is approximately one half to one third that of isolated purified myosin measured by the same technique. The slower rate observed in the myofibrils suggests that interactions within the myofilament array act to inhibit the rate of ATP turnover compared with that of the purified protein. This inhibition may play an important role in regulating the rate of thermogenesis in living mammals. If the rate of purified myosin, measured at the body temperature of the rabbit, occurred in vivo, the ATP turnover by myosin alone would account for more heat output than is observed in living muscle fibers. Thus, the down-regulation of the cycling rate of myosin heads in relaxed muscle is an important requirement for maintaining the appropriate total energy expenditure of the animal. Alterations in this regulation may also play a role in maintaining homeostasis of body temperature.

METHODS

Myofibrils were prepared from white rabbit muscle by the method of Etlinger, Zak, and Fischman (1976), with an inhibitor cocktail consisting of 0.1 mM PMSF, 0.01 mM leupeptin, and 100 units/ml Aprotin and were stored in 50% glycerol, 50% rigor solution at −20°C and used within 3 mo. Myofibrils were prepared daily from stored myofibrils. Myofibrils in glycerol were diluted 10-fold in rigor solution (120 mM potassium acetate (KAc), 60 mM MOPS, 5 mM EGTA, and 5 mM $MgCl_2$ at pH 7.0) and spun at a relative centrifugal force of 12,000 *g* for 10 min, resuspended in rigor, homogenized for 10 s, and filtered through a double layer of cheese cloth. This procedure was repeated three times with all steps performed between 0 and 4°C. The myofibril suspensions were between 22 and 25 mg/ml of protein as assayed by the Bradford procedure (Bradford, 1976). The content of myosin in myofibrils was taken to be 45% of total protein (Cooke and Franks, 1980). Myosin was prepared from rabbit back and leg muscles by the method of Tonomura, Appel, and Morales (1966), with the modification that the protease inhibitor cocktail described above was included during all steps. The myosin showed no evidence of proteolysis when analyzed using gel electrophoresis in the presence of SDS, with both heavy chains and light chain bands that were identical to their counterparts found in the myofibrils. Myosin was resuspended in a similar rigor solution, except that [KAc] was lowered to 60 mM to promote better filament formation. The myosin suspensions were between 11 and 12 mg/ml of protein, so that the final myosin content was similar to that in the myofibril suspension. The *N*-methylanthranoloyl derivative of ATP was synthesized according to the method of Hiratsuka (1985). Thin layer chromatography on cellulose polyethyleneimine plates showed that the material was >98% pure. Steady state measurements of myofibrillar ATPase activity were made by a modification of the malachite green method (Kodama, Fukui, and Kometani, 1986; Lanzetta, Alvrez, Reinach, and Candia, 1979). Myofibrils were incubated with 1–3 mM ATP in rigor buffer plus the oxygen scavenger systems in conditions that were identical to those used in the fluorescence experiments, and aliquots were withdrawn at 2, 5, and 10 min and assayed for phosphate.

Fluorescence was measured using a fluorimeter (SPEX Industries, Inc., Edison, NJ) interfaced to an IBM PC-XT, with excitation at 350 nm and emission at 440 nm. Both excitation and emission slits were at 2.5 mm, producing bandwidths of 10 nm. Fluorescence emission was collected using the front face optical path from cells that were 2 mm thick. The optical density of the most turbid samples, 10 mg/ml myofibrils, was 0.9 at a wavelength of 350 nm and 0.6 at a wavelength of 440 nm.

To measure ATP turnover, a sample containing myofibrils or myosin relaxed in rigor solution plus ATP (as described below) was rapidly mixed (mixing time, ~1 s) with an equal volume of a solution containing mant-ATP in a home-built apparatus and injected into the fluorescence cuvette (Fig. 1). The change in the fluorescence of mant-ATP was then measured as a function of

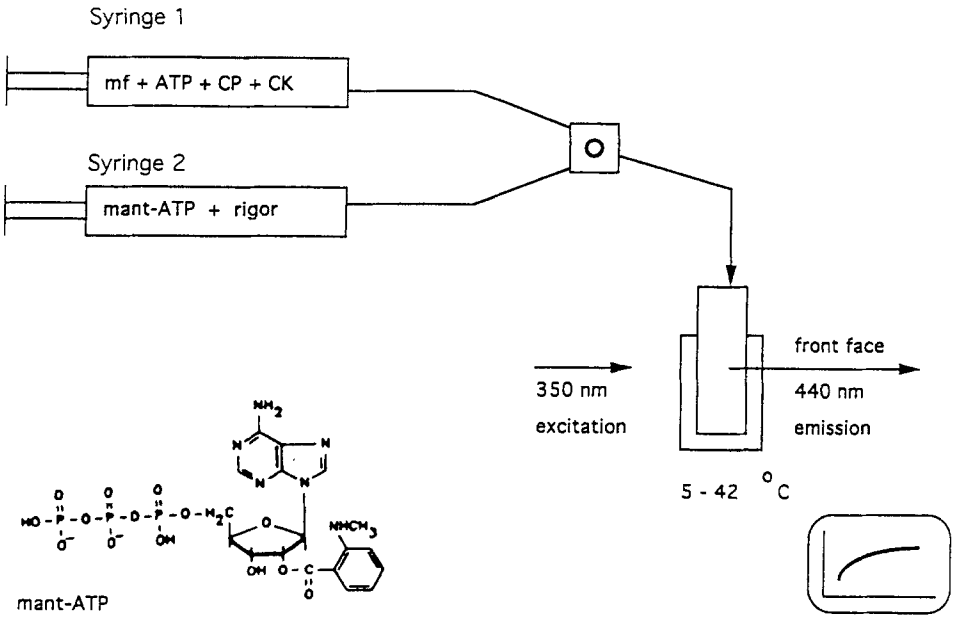

FIGURE 1. A diagrammatic representation of the experimental procedure, showing the rapid mixing of the contents of two syringes just before injection into a quartz cuvette in a fluorimeter, and the computerized measurement of the fluorescence intensity over time. Syringe 1 contained relaxed myofibrils with a high concentration of ATP (typically 1.6 mM) and syringe 2 contained a solution with a low concentration of mant-ATP (typically 80 μM). The diagram of mant-ATP shows the fluorophore attached to the 2′ position on the adenine ring; however, this group isomerizes rapidly with the 3′ position.

time. The myofibrils were relaxed in rigor solution with high concentrations of ATP, 0.4–10 mM, and an ATP regenerating system consisting of 1 mg/ml creatine kinase, and 20 mM creatine phosphate (CP). An oxygen scavenging system of glucose (10 mM), glucose oxidase (0.03 mg/ml), and catalase (0.02 mg/ml) was added before the myofibrils were drawn up into a syringe (Harada, Sakurada, Aoki, Thomas, and Yanagida, 1990). The syringe tip was closed and the syringe was preincubated in a temperature-controlled water bath for 10 min. Similarly, a second syringe containing rigor buffer, a low concentration of mant-ATP, and oxygen scavengers was covered with foil to prevent photodecay of the fluorescent nucleotide and also incubated for 10 min. The contents of the two syringes were then rapidly mixed and injected into a quartz cuvette in a temperature-con-

trolled fluorimeter. The temperature was monitored with a thermistor probe. The final concentrations of ATP and mant-ATP in the cuvette varied but in standard conditions were 0.8 mM or 1.6 mM and 40 μM, respectively. The oxygen scavengers were necessary to prevent photo-oxidation of the mant-ATP during the assay. In its absence, there was a linear decrease in the fluorescence intensity during the assay, which complicated the interpretation of the data.

As ADP dissociated from the myosin in the myofibrils, ATP and mant-ATP competed for the nucleotide binding site and because the fluorescence of mant-ATP increases on binding to the myosin head, the rate of mant-ATP binding could be monitored over time. The scan time was typically between 3 and 8 min (depending on the preparation and the assay temperature) by which time a plateau in the fluorescence emission had been reached. The data were fit with a single exponential to determine the rate of nucleotide turnover:

$$y = I_o + I_{inf} \left[1 - \exp\left(-k_{app} \cdot t\right)\right], \tag{1}$$

where I_o, I_{inf}, and k_{app} were the three parameters varied in the fit, respectively, representing the background fluorescence, the amplitude of the signal that arises from the slow binding of the analogue to sites in the sample, and the rate associated with this binding. In some cases, there was evidence of a decrease in the fluorescence intensity after the steady state plateau had been reached. This can probably be attributed to photodecay. In these instances the data were fit with a straight line in addition to the above two terms. The rate at which the emission intensity approached a steady state and the amplitude of the signal were determined from the solved equations. We also measured MgATP turnover rate for myosin by the same method.

Two types of control experiments were done: one in which myofibrils were not incubated with ATP but remained in rigor until mixed with an ATP and mant-ATP solution from syringe 2, and another in which 5.1 mM of Ca^{2+} was added with the mant-ATP solution from syringe 2.

We did a number of experiments in which we varied the assay conditions. The temperature was varied between 5° and 42°C. At 25°C, we determined the effect of varying the final [ATP] between 0.24 mM and 5 mM while maintaining the final [mant-ATP] at 0.04 mM. We also varied the [KAc] in the rigor buffer between 0.12 and 0.18 mM. The effect of changing pH from 7 to 6.2 was measured at 10 and 20°C. The effect of adding 25 mM Pi was also determined at 10 and 20°C. In the latter experiments, the [KAc] was reduced to 0.11 M to maintain a constant ionic strength, and the results were compared with control experiments at 0.18 M KAc.

The binding of mant-ATP provides an accurate determination of the rate of nucleotide turnover in the sample. The release of the products of hydrolysis is slow, and the rebinding of ATP or mant-ATP is very fast. Mant-ATP will replace a fraction of the bound nucleotides as they are released, and the fraction depends on the relative association rates and the relative concentrations of the mATP and ATP. Assuming that the binding of nucleotides can be described by a second-order rate constant and their release by a single first-order rate constant, we obtain the following kinetic scheme, where M = myosin, N = nonfluorescent nucleotides, mN = mant nucleotides:

$$\mathrm{M.N} \underset{k_-}{\overset{k_+ \; N}{\rightleftharpoons}} \mathrm{M} \underset{k_{m-}}{\overset{mN \; k_{m+}}{\rightleftharpoons}} \mathrm{M.mN} \cdot \tag{2}$$

At time = 0, all myosin is in the M.N state. As myosin releases its bound nucleotide, it rebinds either mN or N, which is subsequently released with their corresponding off rates. A straightforward analysis of this scheme leads to an expression that predicts an exponential incorporation of mN into myosin with an apparent rate constant, k_{app}, given by:

$$k_{app} = k_+ [N] \cdot k_{m-} / (k_{m+} [mN] + k_+ [N]) + k_{m+} [mN] \cdot k_- / (k_+ [mN] + k_+ [N]), \tag{3}$$

and a final extent of incorporation I_{inf} given by:

$$I_{inf} = (k_{m+}[mN]/k_{m-})/(k_{+}[N]/k_{-} + k_{m+}[mN]/k_{m-}). \quad (4)$$

Both the apparent rate constant and the final extent depend on the on rates and the off rates of the two nucleotides. The off rate of the mant nucleotides from myosin has been shown to be the same as for ADP from myosin, whereas the second-order rate constant for binding mant-ATP is about three times greater than that of ATP (Woodward et al., 1991). If this is also true in the fibers, the value of I_{inf} should depend upon the relative concentrations of the two nucleotides and their respective rates as given by Eq. 4. We show in Results that this is in fact found. Assuming then that $K_{m-} = k_{-}$, Eq. 3 reduces to:

$$k_{app} = k_{-}. \quad (5)$$

Thus, the measured rate of incorporation of the mant nucleotides is equal to the rate of ADP turnover by myosin.

In the above we make the assumption, justified below, that the concentrations of the two nucleotides are constant during the run. For a typical run, the final concentrations of ATP, mant-ATP, and myosin active sites were 800, 40, and 20 μM, respectively. The second-order rate constant for binding mant-ATP is about three times greater than that of ATP (Woodward et al., 1991). Thus, the ratio of the relative rates for binding ATP and mant-ATP is ~800/40·3 = 6.7. Therefore, upon reaching steady state, only 15% of the myosin heads will have bound mant-ATP and only 7% of the mant-ATP will have bound to the proteins, the rest of the mant-ATP remaining in solution. Mant-ADP is a substrate for creatine kinase, thus the mant-nucleotides remained in the triphosphate form in the sample (Hiratsuka, 1983). This was verified by analysis of the nucleotide using thin-layer chromatography after data collection. The fact that the increase in fluorescence intensity can be well fit by a single exponential also shows that the rate of mant-ATP binding has not been altered during the time course of a single assay. Observation of the myofibrils at the end of the run showed that their sarcomere length (2.2–2.4) had not shortened during the measurement of fluorescence in the absence of Ca^{2+}. The state of myosin phosphorylation was determined via isoelectric focusing. The regulatory light chain of purified myosin after an ATPase assay showed little phosphorylation, 5–10%. The regulatory light chains in the myofibrils showed a slightly greater level, 10–20%. Data are presented as means ± SEM.

RESULTS

Measuring the Rate of Nucleotide Turnover Using a Fluorescent Nucleotide

Fig. 2 represents a typical trace of the fluorescence emission measured over the time of the experimental procedure. At $t = 0$ the relaxed myofibrils were mixed with mant-ATP. The fluorescence intensity of mant-ATP is increased in nonpolar environments, thus the binding of mant-ATP to proteins results in an increase in the observed intensity (Hiratsuka, 1983). Binding to active myosin heads, or to most other enzymes in the preparation, will occur in less than a second, before the trace shown in Fig. 2 begins. The slow increase in intensity shown in Fig. 2 indicates that some protein binds the mant-ATP very slowly. A number of experiments, discussed below, show that this slow binding of the fluorescent nucleotide can be attributed to relaxed myosin heads in the myofibrils. As ADP is released from the myosin heads in the myofibrils, mant-ATP is able to compete with ATP for a portion of the nucleotide binding sites, and it is incorporated into the myosin active site at the rate at which the nucleotides are turning over. The concentrations of ATP and

mant-ATP are far in excess of that required to fully relax the myofibrils, and the concentration of mant-ATP is in excess of that of the concentration of myosin heads. Thus, the plateau represents the point at which the ratio of bound mant-ATP to bound ATP has reached a steady state. Fig. 2 also shows the data fitted with a single exponential, Eq. 1, with terms I_o and K_{app} determined. The value of r^2 was 0.99 indicating that a single exponential provided an excellent fit, as is also evident from the lower trace, which represents the residual. A fit to the data with a double exponential equation did not provide a significantly better value of r^2, thus, the single exponential equation was subsequently used throughout.

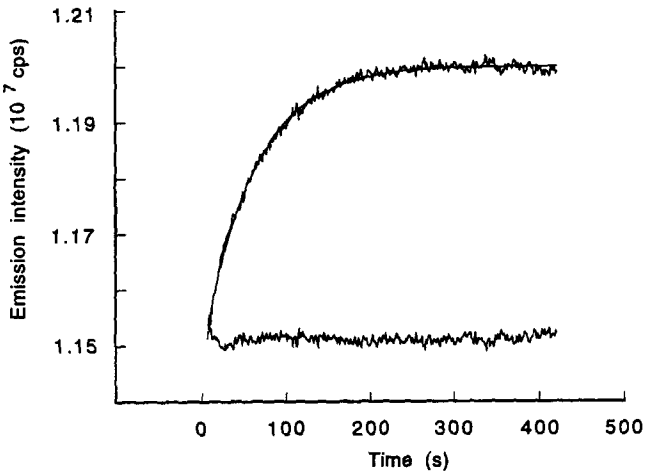

FIGURE 2. The upper trace shows fluorescence intensity increasing over time until a steady state was reached. The final concentrations were myofibrils, 10 mg/ml; mant-ATP, 40 μM; and ATP, 0.8 mM. The temperature was 20°C and the scan time was 7 min. The data were fit with a single exponential equation: $y = I_o + I_{inf}[1 - \exp(-k_{app} \cdot t)]$. The equation was resolved with $I_o = 1.15$ 10^7 cps, $I_{inf} = 4.86$ 10^5 cps, and the rate constant $k_{app} = 0.017$ s^{-1}. The r^2 value indicating the goodness of fit was 0.99. The lower trace, which represents the residual between the data and the fitted curve, shows that the increase in fluorescence intensity is well described by a single exponential.

Two parameters can be measured from traces such as that shown in Fig. 2: the amplitude of fluorescence increase and the rate of approach to the steady state. The amplitude reflects the fraction of the mant-nucleotides that become bound to proteins in the preparation, and the value expected from binding to myosin can be calculated from known parameters. The second-order rate constant for the binding of mant-ATP to myosin has been measured by Woodward et al. (1991), who found a value of 3.6×10^6 M^{-1} s^{-1}. This is 3.6 times that of ATP, showing that the fluorescent moiety actually promotes faster binding. In Fig. 2 the final concentrations of ATP, mant-ATP, and myosin sites were 800, 40, and 20 μM, respectively. The steady state concentration of bound mant-nucleotide can be shown from Eq. 4 to be equal to 3 μM. Upon binding, the fluorescence intensity increases 2.6-fold, leading to a

calculated increase in the observed intensity of 9.6%. This is close to the observed value of 8.2% ± 0.8% (n = 20), providing evidence that the observed increase in fluorescence intensity arises from binding to myosin in the myofibrils. Further evidence supporting this conclusion came from traces obtained at a higher concentration of ATP. In the presence of 5 mM ATP, the rate of the fluorescence increase was the same but the amplitude was smaller, 2.6% ± 0.4% (n = 18). This observed value again compares favorably with the calculated value from Eq. 4 of 2.2%, showing that ATP can compete for the sites responsible for the increase in fluorescence. This can be clearly seen in Fig. 3, where the top trace was obtained from an experiment with an [ATP]/[mant-ATP] of 800:40 μM, whereas in the bottom trace, the

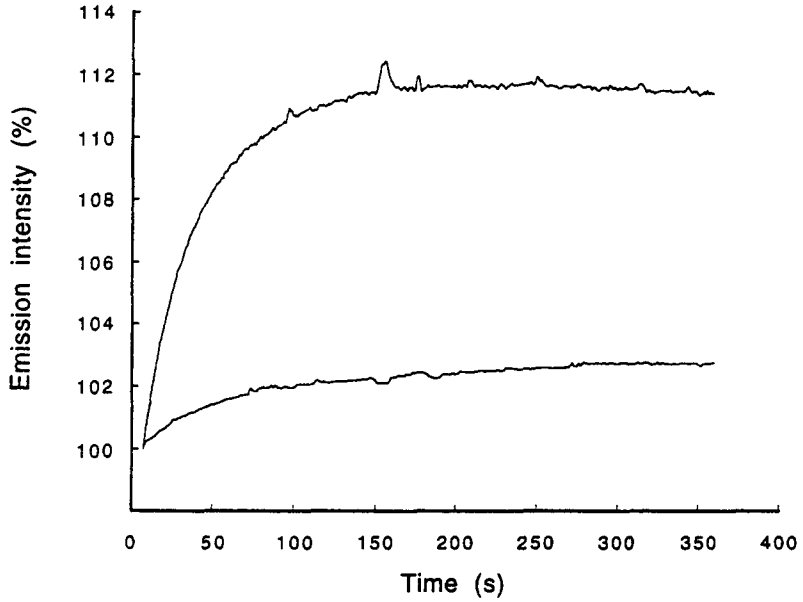

FIGURE 3. Two typical traces of fluorescence intensity increasing over time. The data were normalized to reflect a percentage of increase over a baseline initial emission intensity, which was chosen as the earliest point on the exponential rise. The upper trace represents a final [ATP] of 0.8 mM and the lower a final [ATP] of 5 mM. In both traces the final [mant-ATP] was 0.04 mM. In the higher ATP, the increase in intensity has a smaller amplitude, but follows the same time course (0.027 s^{-1}).

ratio was 5 mM:40 μM. In Fig. 3 the calculated nucleotide turnover rates were both 0.027 s^{-1}. The mean rate of fluorescence increase, determined after fitting each scan to a single exponential, was 0.03 ± 0.001 s^{-1} at 25°C (n = 56). This rate is approximately a factor of two lower than the rate for myosin alone measured by the fluorescence assay at the same temperature (25°C) (0.054 ± 0.003 s^{-1}; n = 10). The slower rate of nucleotide turnover cannot be attributed to denatured protein in the myofibrils or to an overestimate of the fraction of myosin, because the rate is measured from an approach to steady state and is not dependent upon the actual concentration of active sites.

Although the total change in fluorescence intensity is small, of the order of 2–10%, the signal-to-noise ratio is large, of the order of 50. Thus, the time course of the binding of the fluorescent nucleotide to the proteins or myofibrils can be measured with great precision. The small size of the signal did, however, present some problems. The intensity of fluorescence increases with decreasing temperature, with a 1% change in intensity occurring per degree, between 10 and 20°C. This necessitated rigorous temperature control, including incubation of the samples before mixing, precise control of the temperature of the mixing apparatus and of the cuvette. Measurement of the temperature of the sample in the cuvette during several runs showed that temperature changed by less than 0.5°C, providing sufficient control to eliminate this potential artefact. Particularly at ratios of [ATP]/[mant-ATP] of 800:40 μM, which was the most common experimental condition, the potential error would be <10%.

We did three control experiments to provide further assurance that we were in fact measuring the displacement of ADP from the myosin head by mant-ATP. In the first we added the ATP and the mant-ATP together to myofibrils in rigor buffer. In this case, the two nucleotides reach steady state binding ratios immediately upon mixing, which resulted in a steady value of fluorescence at a similar emission intensity as the plateau reached in the experiments that followed the protocol of Fig. 2. This result indicates that there was little binding of mant-ATP to nonspecific sites, such as membranes, that might also enhance intensity. For the second control, we added calcium with the mant-ATP, so that steady state active turnover was set up immediately. A similar result was obtained with a constant emission intensity. For the third we added an inhibitor of the myosin ATPase activity, butane dione monoxime (BDM) (Herrmann, Wray, Travers, and Braman, 1992*b*). The rate of nucleotide turnover decreased to 58 ± 5 s^{-1} upon addition of 5 mM BDM to the relaxed myofibrils at 25°C. Thus, the BDM has altered the turnover rate by a factor of 1.8, which is close to the factor of 2.2 obtained by Herrmann et al. (1992*b*) for inhibition of the myosin ATPase activity by 5 mM BDM. Thus, all three experiments suggest that the observed increase in intensity arises from the binding of mant-ATP to the ATPase site of myosin.

The rate of nucleotide turnover observed using the fluorescence assay, 0.030 ± 0.001 s^{-1} at 25°C, was several times slower than the steady state rate measured from phosphate production in unactivated myofibrils from the same preparations—0.26 ± 0.03 s^{-1}. This result shows that the rate observed by steady state measurements is increased over the true rate of a fully relaxed myosin head. The difference can be attributed both to other more rapid enzymes or to active cycling of myosin heads in regions where the regulatory proteins have been lost or damaged. Because the rate for active myofibrils, ~2 s^{-1}, is almost 100 times faster than for relaxed myofibrils, the observed difference could be accounted for by a small fraction, 1%, of active myosin heads.

The Effect of Varying Conditions on the Nucleotide Turnover of Fully Relaxed Myofibrils

We determined the effect of varying the final ATP concentration, from 0.2 to 5.0 mM, on the rate of nucleotide turnover. The nucleotide turnover rate decreased as

the substrate concentration increased from 0.14 ± 0.03 s^{-1} at 200 μM ATP to 0.051 ± 0.004 s^{-1} at 5 mM ATP at 35°C, and at 25°C from 0.037 ± 0.003 s^{-1} at 400 μM ATP to 0.031 ± 0.002 s^{-1} at 5 mM ATP. The fully relaxed rate was only achieved near 0.8 mM final ATP, (0.030 ± 0.002 s^{-1}), and above this the rate remained constant. The apparent K_m for relaxation can be estimated from the nucleotide concentration at which the observed rate has been doubled, ~300 μm. This value is much greater than observed for the ATPase activity of myosin, ~0.05 μm (Taylor, 1979). The difference between these values shows that full relaxation of the myofibrils requires virtually complete saturation of myosin by ATP. A very few myosin heads that do not bind ATP can bind to the thin filament, causing local activation of the filament and rapid cycling of adjacent myosin heads. The observation that the rate of mant-ATP binding increased with decreasing substrate can be analyzed in terms of the known kinetic constants (see Discussion).

Various concentrations of KAc in the rigor solution did not affect the slow nucleotide turnover measured in this system. At 25°C the nucleotide turnover rates were 0.033 ± 0.001 s^{-1} (n = 6), 0.029 ± 0.001 s^{-1} (n = 32), and 0.032 ± 0.003 s^{-1} (n = 19) at 0.12 M, 0.15 M, and 0.18 M KAc, respectively.

It is known that several factors, such as pH or [Pi], which change during fatigue, influence the nucleotide turnover rate of active fibers (for a review see Fitts, 1994). To determine whether these factors also affected the turnover rate of relaxed fibers we measured the effects of decreasing the pH from 7 to 6.2, or increasing the phosphate concentration from 0 to 25 mM. When Pi concentration was increased, the concentration of KAc was decreased to 0.11 M so that ionic strength was maintained at the same level as in control experiments. An increase in phosphate concentration did not alter the nucleotide turnover rate from control conditions (94.7% ± 6.5%, n = 7). Decreasing the pH from 7 to 6.2 reduced the nucleotide turnover rate by a factor of almost 2 (to 63.3% ± 5.1% of the value at pH = 7, n = 11).

The Effect of Temperature on the Nucleotide Turnover Rate of Fully Relaxed Myofibrils

We measured the effect of temperature on the nucleotide turnover rate of both myofibrils and purified myosin between 5 and 42°C. The time constants for the rate of fluorescence emission increase in myofibrils is presented as a function of temperature in Fig. 4 *A*. The time constant decreased from 93.8 ± 7.0 s at 5°C (n = 7) to 15.3 ± 1.6 s (n = 12) at 42°C. The corresponding time constant for myosin was 29.8 ± 3.0 s (n = 6) seconds at 10°C, decreasing to 9.7 ± 0.9 s (n = 9) at 35°C. Steady state measurements of myofibrillar ATPase rate using conventional methods showed that the rate was 0.034 ± 0.008 s^{-1} at 10°C, 0.126 ± 0.033 s^{-1} at 20°C, and 1.13 ± 0.134 s^{-1} at 40°C. These rates are considerably faster than those observed by the fluorescent method. The value of Q_{10} (the multiplicative increase in rate for a 10°C change in temperature) was 1.66 ± 0.03 for myofibrils measured by the fluorescent technique. The value for the myosin data, shown in Fig. 4 *B*, gives a Q_{10} of 1.8. When plotted as an Arrhenius plot (see Fig. 4 *B*), the energy of activation for the myofibrillar ATPase activity is calculated to be 33 ± 8 kJ/mol, which is less than that obtained from the combined values for purified myosin, shown in Fig. 4 *B*, 38 kJ/mol. The linearity of this plot provides assurance that the assay and the proteins

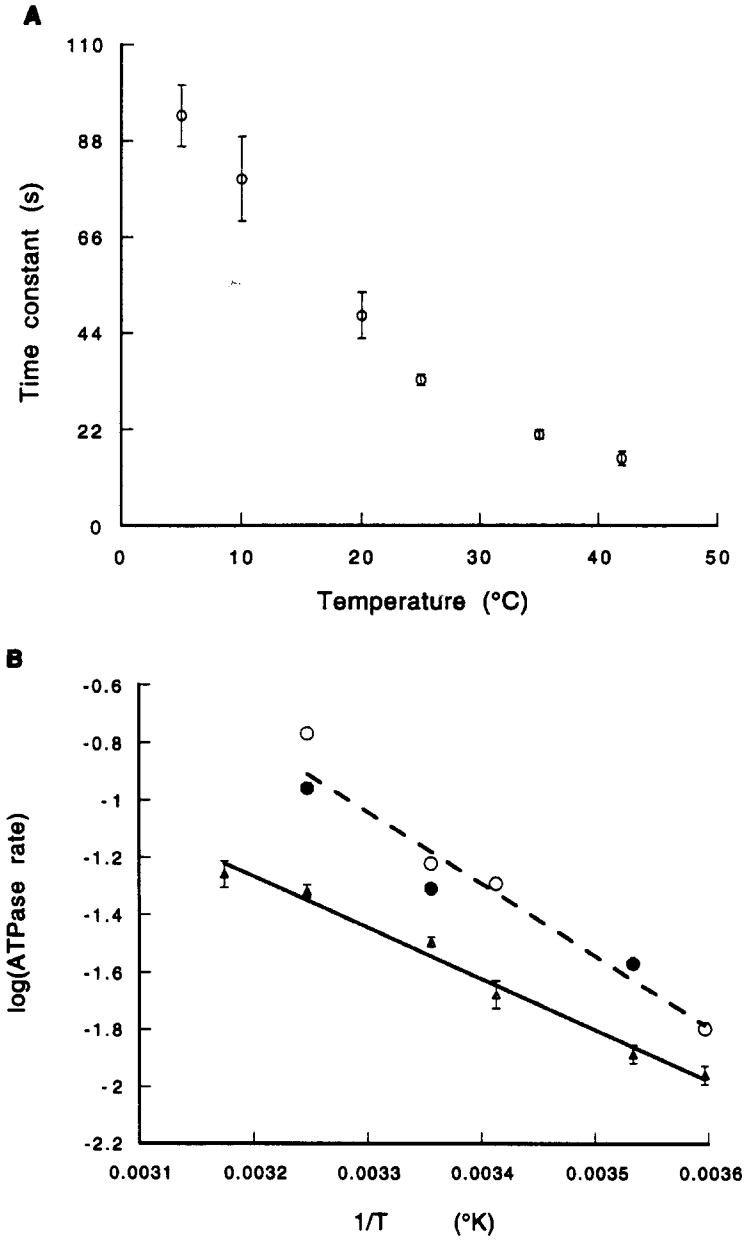

FIGURE 4. (*A*) The time constant of nucleotide turnover by relaxed myofibrils is shown as a function of the temperature. The data were collected as shown in Fig. 2 with a final concentration of ATP between 0.8 and 5.0 mM and mant-ATP = 40 μM. (*B*) Arrhenius plots, $\log_{10}$ turnover rate vs 1/temperature, for the data shown in Fig. 4 *A* (△) and for myosin assayed by our new method (●) and by other methods (○) (Bagshaw et al., 1972; Bagshaw and Trentham, 1974; Taylor, 1979; Herrmann et al., 1992). The slopes of the lines define an activation enthalpy of 38 ± 3 kJ/mol for myosin and 33 ± 1 kJ/mol for myofibrils, where the errors reflect the 95% confidence limits on the slopes.

work as expected at the higher temperatures. At 39°C, the in vivo temperature of the rabbit, the turnover rate for purified myosin is $0.15 \pm 0.04\ s^{-1}$ and that for the myofibrils is $0.04 \pm 0.005\ s^{-1}$.

DISCUSSION

We have shown that it is possible to measure the ATPase rate of relaxed myofibrils by mixing them rapidly with a fluorescent ATP analogue. It provides a method for observing the slow rate of nucleotide turnover in the presence of more rapid turnover by other enzymes and by small populations of active myosin heads. The method requires a relatively simple mixing apparatus because the time course of the reaction is so slow. It provides a real-time method of observing a single turnover of the bound nucleotides. Because the ratio of native substrate to nucleotide analogue is high, only a small portion of the myosin heads actually bind the fluorescent nucleotide, thus its presence provides a minimal perturbation to the system. Therefore, although the fluorescence of a nucleotide analogue is observed, the rate that is measured is equivalent to that for the native substrate.

Several results argue that the rise in intensity which we observed is the result of the binding of the analogue to the active site of myosin. The rates for purified myosin alone are similar to those measured by standard methods (Bagshaw, Eccleston, Trentham, and Yates, 1972; Bagshaw and Trentham, 1974; Taylor, 1979). In the myofibrils the change in the intensity of the analogue is approximately that which can be calculated from the known change in the quantum yield of the analogue upon binding to myosin and the known relative second-order rates of binding of the analogue and ATP. The amplitude of the change in intensity is diminished as the concentration of the nucleotide is increased, with the 2.6% change found at 5 mM ATP almost exactly that which is calculated from the relative rates of binding of ATP and analogue. Thus, the increase in the intensity is the result of binding to a site that is specific for ATP. Protocols in which the myosin heads cycled rapidly, or in which the steady state was achieved rapidly, eliminated the slow increase in intensity. The presence of a known inhibitor of the myosin ATPase activity, BDM, has the effect predicted from studies of purified myosin (Herrmann et al., 1992*b*). Finally, when the ATP is omitted from the syringe containing the myofibrils, the binding of the low concentration (40 μM) of fluorescent nucleotide is rapid, and the slow rise in fluorescence intensity is abolished, demonstrating the requirement of initial ATP to ensure slowly cycling, relaxed cross-bridges.

As the concentration of ATP is decreased, the rate of the fluorescence intensity change becomes faster. This phenomenon is also predicted from the known kinetics of the interaction of myosin heads with myosin. The presence of rigor cross-bridges displaces the regulatory proteins from their inhibitory position, allowing adjacent cross-bridges to cycle actively (Bremel and Weber, 1972). Because the rate of cycling by active cross-bridges is so much faster than that of the relaxed cross-bridges, the presence of only a few rigor bridges will influence the observed rate. The observed increase in the rate can be explained approximately by the known K_m for myosin and the relative rates for the cycling of active and relaxed myosin heads. At a concentration of 300 μM ATP the activity of the relaxed myofibrils has in-

creased by a factor of two, compared with that at higher [ATP], or by 0.03 s^{-1}. At this concentration, assuming a K_m of 0.05 μM for the myosin ATPase activity, the fraction of rigor myosin heads would be $K_m/[ATP] = 1.6 \times 10^{-4}$. If each active head cycled at 20 s^{-1} then the observed increase in the resting ATPase activity could be explained if each rigor head induced ~10 active cycles in adjacent myosin heads. Considering that a rigor head could displace a tropomyosin controlling seven actins, this is not unreasonable.

Together, the results described above provide assurance that the increase in the intensity of the fluorescence is the result of replacement of the adenine nucleotides from myosin. In theory, these nucleotides could take two routes in dissociation from the myosin head, ADP could dissociate after ATP hydrolysis, or ATP could dissociate before hydrolysis. The rate of ATP dissociation from relaxed myofibrils has been shown to be slower than our observed fluorescence change, showing that the first path described above is the major one measured (Herrmann, Houadjeto, Travers, and Barman, 1992*a*). Thus, the method described in this paper provides a simple and accurate measurement of the rate of cycling of myosin heads in relaxed myofibrils.

Two important observations are provided by this assay. The first is that the rate of nucleotide turnover found by the fluorescence trace is slower than steady state measurements on the same myofibrils. This difference can be attributed to other enzymes or to damaged and active portions within the myofilament array. The second is that the activity of the myofibrils was considerably less than that of the purified protein. The difference found here is greater than that observed by Herrmann et al. (1992*a*) from steady state measurements. This difference shows that some mechanism associated with the structure of the filament array acts to inhibit the ATPase activity of the myosin head. The activation energies, calculated for myofibrils and for myosin from the Arrhenius plots shown in Fig. 4 *B*, are different by more than two standard errors. This observation suggests that the rates are determined by two different steps, again indicating that interactions within the filament array alter the ATPase activity compared with that in the purified and isolated protein. The difference in activation energies produces a greater degree of inhibition as the temperature increases, reaching almost a factor of three at 39°C.

The mechanism that is responsible for the inhibition remains unknown. However, it is probable that it involves some interaction between the myosin head and the core of the thick filament. In smooth muscle myosin there is an interaction between the regulatory light chain and the tail region that greatly inhibits nucleotide turnover, producing an enzymatically inactive state (Cross, Cross, and Sobieszek, 1986). The regulatory light chain of skeletal myosin is known to interact with the core of the thick filament, which is composed of the myosin tail regions (Roulet, Burgat, and Cardinaud, 1993). Together these two results suggest the hypothesis that an interaction between the regulatory light chain and the tail region may occur in skeletal myosin and regulate catalytic activity. This interaction would require a native thick filament because it is not observed for precipitated purified myosin.

If the hydrolysis rates observed for myofibrils or for myosin occurred in the living fiber, they would lead to a heat production that can be easily calculated. The en-

thalpy of metabolism of glucose is 470 kJ mol^{-1} per molecule of O_2 (see Homsher and Kean, 1978, for discussion). Assuming an ATP/O ratio of 6.2:1, the heat associated with the production and turnover of one molecule of ATP is calculated to be 72 kJ/mol. The concentration of myosin in living fibers has been measured to be 280 μM (Ebashi, Endo, and Ohtsuki, 1969). Thus, the turnover rate for purified myosin 0.16 s^{-1} at 39°C, obtained from the fit to all the data shown in Fig. 4 *B*, would lead to a rate of heat production of $280 \times 10^{-6} \times 72 \times 10^3 \times 0.16$ J $liter^{-1}$ = 3.2 J $liter^{-1}$ s^{-1}. The rate observed for the relaxed myofibrils is 1.2 J $liter^{-1}$ s^{-1}, approximately one third the rate observed for purified myosin.

The rate of heat production that would be generated by our observed ATPase activities, calculated above, can be compared with that observed for living muscle. The rate of O_2 consumption measured for resting muscle, 2.1 ml kg^{-1} min^{-1}, implies a resting heat production of 0.72 J $liter^{-1}$ s^{-1} (Gutierrez, Pohl, and Narayana, 1989). Although this is the only measurement we found for rabbit muscle, the value can also be inferred from measurements on other mammals. The rate of heat production for human muscle in situ is 0.5 J $liter^{-1}$ s^{-1}, and the average whole body basal rate is 1.4 J $liter^{-1}$ s^{-1} (Astrup, Simonsen, Bülow, Masdsen, and Christensen, 1989; Kelly, Reilly, Veneman, and Mandarino, 1990; Zurlo, Larson, Bogardus, and Ravussin, 1990; Blei, Conley, and Kushmerick, 1992). The basal resting heat production for rabbits is 2.5 J $liter^{-1}$ s^{-1} (Schmidt-Nielsen, 1970). Assuming that the ratio of muscle heat production to whole body heat production is the same in the two mammals, the rate of heat production in rabbit muscle is calculated to be 0.9 J $liter^{-1}$ s^{-1}. Additional estimates for heat production by rabbit skeletal muscle were obtained from scaling the observed rates of heat production from dogs, (1.97 J $liter^{-1}$ s^{-1}), cats, (0.53 J $liter^{-1}$), and rats (1.5 J $liter^{-1}$ s^{-1}) (Chapler, Stainsby, and Gladden, 1980; Chinet, Decrouy, and Even, 1992; Kushmerick, Meyer, and Brown, 1992). The average basal heat production in rabbit muscle obtained by scaling the results of eight different measurements was 1.0 ± 0.3 J $liter^{-1}$ s^{-1}. Thus, it appears that the basal heat production in resting rabbit is close to 0.7–1.0 J $liter^{-1}$ s^{-1}.

The measured nucleotide turnover rate for purified myosin alone would imply a higher basal rate of heat production or O_2 consumption than is observed for muscle. The observed rate of heat production of 0.7–1 J $liter^{-1}$ s^{-1} estimated above for rabbit muscle in situ is only ~20–30% of that which would be expected if each myosin head hydrolyzed ATP at the turnover rate observed for purified myosin. If the myosin heads in living rabbit muscle cycled at the rate observed for purified myosin at the body temperature of a rabbit, the nucleotide turnover of relaxed myosin would account for ~50% of the total energy expenditure of the resting animal. The heat production by the ATPase activity of the myosin heads must be considerably less than the rate of heat production of living muscle, when one considers that myosin turnover is only one pathway that will use ATP. Maintenance of ionic gradients, production of new proteins, etc., would be expected to also contribute to the rate of heat production. The contribution of maintaining intracellular gradients of calcium has been estimated to require as much as 40% of total basal energy expenditure (Chinet et al., 1992). The fact that muscle has a lower basal rate of heat production than most other tissues, with a rate that is only one third that of the whole organism, suggests that much of the basal energy expenditure by this tissue must be

used for cellular homeostasis. Thus, the contribution owing to nucleotide turnover by the relaxed myosin heads must be considerably less than the upper limit determined above from the total basal heat output. It is interesting that upon hypoxia, the rate of O_2 consumption in rabbit muscle decreases by a factor of 10, although ATP levels have remained close to normal (Gutierrez et al., 1989). This would imply an even more dramatic upper limit to the allowed ATPase activity by myosin under these conditions.

Several results suggest that thermogenesis by muscle may play an important role in maintaining proper basal body temperature. Although, the rate of thermogenesis by muscle is lower than that of other tissues, muscle constitutes ~40% of the mass of the average mammal, so that the total contribution is appreciable (15–20%) (Cossins and Bowler, 1987). The resting metabolic rate among different people has been shown to correlate well with the basal rate of energy expenditure by skeletal muscle (Zurlo et al., 1990). Thus, difference in the basal economy of muscle may account for part of the variance among individuals. Skeletal muscle has been shown to be the major site for nonshivering thermogenesis in cold-acclimated ducklings (Duchamp and Barré, 1993). Thus, mechanisms that regulate thermogenesis in muscle may play an important role in the energy economy of the animal.

From the discussion above one can conclude that some mechanism acts to inhibit the turnover rate for the myosin head in living mammalian muscle. A similar conclusion is reached when the heat production observed for living frog muscle is compared with the turnover rate measured for purified frog myosin (Ferenczi et al., 1978*a*). Here the discrepancy is even greater than that found for the mammalian muscle, with the rate of heat production by the myosin ATPase activity about five times that observed in the living muscle. Our observation that the turnover rate for myofibrils is slower than that for purified myosin can account for at least a portion of the difference between the living muscle and the in vitro measurements of the rate of nucleotide turnover. The rate of heat production by living rabbit muscle, 1.0 J liter^{-1} s^{-1} is only a little less than that which can be calculated for the heat production caused by the myosin turnover rate observed for the myofibrils, 1.2 J liter^{-1} s^{-1} at the body temperature of the rabbit. Thus, the incorporation of the myosin into the filament array of the myofibril substantially inhibits the myosin ATPase turnover rate when measured in fully relaxed preparations, and explains to some extent the low resting metabolic rate in mammals. However, when one considers that turnover by myosin must be only one of many reactions that consume ATP in living muscle, it is clear that the actual turnover rate in living tissue must be even slower than that observed for the myofibrils in vitro. It is possible that portions of the regulatory mechanism, which provide the further inhibition seen in the filament array, may have been lost or damaged by the procedures used to produce the myofibrils. Further work using intact skinned fibers or more gentle production of myofibrils can explore this possibility.

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