Mechanochemical Coupling in Muscle: Attempts to Measure Simultaneously Shortening and ATPase Rates in Myofibrils

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ABSTRACT We studied the ATPase of shortening myofibrils at 4°C by the rapid flow quench method. The progress curve has three phases: a P_i burst, a fast linear phase k^F of duration t_B, and a deceleration to a slow k^S. We propose that k^F is the ATPase of myofibrils shortening under zero external load; at t_B shortening and ATPase rates are reduced by passive resistance. The total ATP consumed during the rapid shortening is ATP_c. Our purpose was to obtain information on the myofibrillar shortening velocity from their ATPase progress curves. We tested t_B as an indicator of shortening velocity by determining the effects of different probes upon it and the other ATPase parameters. The dependence of t_B upon the initial sarcomere length was linear, giving a shortening velocity close to that of muscle fibres (V_o). The K_m of ATP was larger for t_B than for k^F, as found with fibers for V_o and their ATPase. ADP and 2,3-butanedione monoxime, but not P_i, inhibited t_B to the same extent as V_o. The $\Delta H^\ddagger$ for t_B and V_o were similar. ATP_c was independent of the sarcomere length, implying that the more the myofibrils shorten, the less ATP expended per myosin head per micron shortened. We propose that t_B can be used as an indicator for myofibrillar shortening velocities.

INTRODUCTION

A key problem in muscle contraction is relating the mechanics to the ATPase of the myosin heads. Muscle mechanics are studied by using fibers, but these are not amenable for precise chemical kinetics. Traditionally, actomyosin subfragment 1 (dispersed molecules in solution) has been used as a model for fiber ATPase, but the suitability of this has been questioned (e.g., Goldman, 1987; Herrmann et al., 1994). The myofibril may be a better approximation, and with them chemical kinetics (Ma and Taylor, 1994; Herrmann et al., 1993, 1994, and references cited therein) and preliminary mechanical studies (Friedman and Goldman, 1993, 1994; Bartoo et al., 1993; Colomo et al., 1995) have been carried out. However, these kinetic and mechanical experiments were done separately, often under different conditions and in different laboratories.

The ATPase activity of shortening myofibrils is studied conveniently by the rapid flow quench method (Houadjeto et al., 1991). A typical progress curve for P_i production (free and protein bound) is illustrated in Fig. 1. It consists of three phases: an initial P_i burst (kinetics not shown on the time scale used), an apparently linear phase (defined by the constant k^F) and then a deceleration to a slow linear phase (k^S). This ATPase profile has been confirmed by a novel phosphate-binding protein method (Brune et al., 1994) in which free P_i is determined specifically and continuously. The phosphate-binding protein and rapid flow quench methods are complementary, each with its advantages and disadvantages (Lionne et al., 1995a).

Here we are particularly interested in the transition of the fast to the slow phase. This is not sharp, but as a first approximation we interpret it by a "break" defined by t_B, which is the time at which the two linear phases intercept. Our working hypothesis (as suggested in Houadjeto et al., 1991, and in Lionne et al., 1995b) is that k^F is the ATPase of myofibrils that are shortening under near zero load. At about t_B, the shortening rate (and ATPase activity) is reduced, presumably because of passive resistance. The reduced ATPase k^S is probably a reflection of the destruction of the myofibrillar lattice structure. The final parameter in Fig. 1, ATP_c, represents the total ATP consumed per myosin head for the duration of the rapid shortening phase, i.e., at t_B.

Our working hypothesis is supported by the finding that myofibrils prevented from shortening by chemical cross-linking do not have a deceleration phase in their ATPase progress curves; there is only a single fast ATPase, close to k^F (Herrmann et al., 1993).

The purpose of this work was to link the four chemical kinetic parameters in Fig. 1 to a mechanical parameter, the myofibrillar shortening velocity. As they are unheld, shortening myofibrils presumably mimic muscle fibres that are contracting under zero external load; the speed of shortening of these fibers has been studied extensively (e.g., Edman, 1979). We define this speed of shortening, which was obtained at saturation in ATP, as V_o. The shortening velocity is related to the length of the elementary contractile unit, the sarcomere. An obvious way to measure myofibrillar shortening velocities is to stop the shortening at different times (e.g., in acid) and then to measure the sarcomere lengths under the optical microscope. Whereas optical measurements provide reasonable estimates of initial sarcomere lengths, they are not precise enough for measuring shortening velocities, as already pointed out by Ma and Taylor (1994). Here, we exploit t_B as an indicator of myofibrillar

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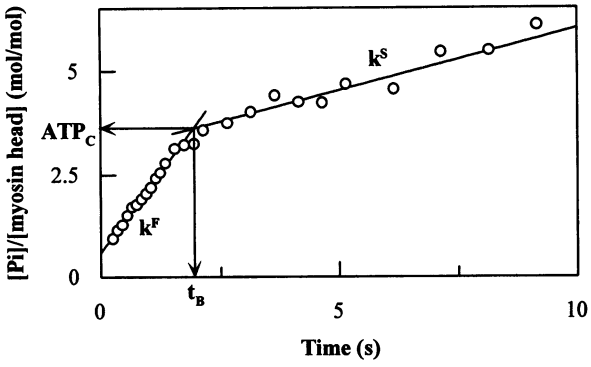

FIGURE 1 Myofibrillar ATPase progress curve obtained by the rapid flow quench method at 4°C. The reaction mixtures (5 μM in myosin heads plus 60 μM [γ-^{32}P]ATP) were quenched in acid at the times indicated and the [^{32}P]P_i determined. For explanations of the parameters k^F, t_B, k^S, and ATP_c and the experimental conditions, see the text.

shortening velocities. Thus, we suggest that if t_B is the duration of the rapid shortening phase, then it is a function of the unloaded shortening velocity of the myofibrils. We put our proposition to the test by determining the effects of the initial sarcomere length, the substrate (ATP) and product (ADP and P_i) concentrations, 2,3-butanedione monoxime (BDM), and temperature on t_B and comparing these with the published data on V_o.

For physicochemists like ourselves this work was a venture. In it we were confronted with the high complexity of an entire biological system (the myofibril is, after all, the contractile unit of the muscle fiber) rather than with the relative simplicity of pure enzymes in solution. We only pretend to provide estimates rather than hard measurements of our parameters.

MATERIALS AND METHODS

Preparation of myofibrils

In our "standard method" myofibrils were prepared from whole rabbit psoas muscle that had been depleted of ATP by leaving the degutted and skinned animal on its back overnight at 4°C. The psoas muscles were removed and myofibrils prepared as in Herrmann et al. (1993). With this type of preparation the myofibrils contained sarcomeres of reasonably homogeneous lengths (2.7 ± 0.1 μm), as found with a large number of preparations (for sarcomere length measurements, see below).

In certain experiments myofibrils containing sarcomeres of different lengths were used. These myofibrils were prepared as described by Knight and Trinick (1982), i.e., by tying slack or stretched psoas muscle fiber bundles to wooden sticks and leaving them overnight at 4°C in rigor buffer (see below). Myofibrils were then prepared from these fiber bundles by our standard method but excluding about 1 cm from the ends of the bundles. The sarcomere lengths in these myofibrils were measured as described below.

Measurement of sarcomere lengths

The sarcomere lengths of myofibrils were measured under a Nomarski microscope (Nikon Microphot, 100×). Typically, the sarcomeres in 20–40 straight myofibrils (chosen at random) containing at least five sarcomeres were measured. The error values are expressed as the standard error of the mean.

It was more convenient to measure directly sarcomere lengths in the muscle fibers used to prepare the myofibrils by laser light diffraction using a 632.8-nm laser, as described by Knight and Trinick (1982). In certain preparations the sarcomere lengths of the myofibrils were checked by optical microscopy, and the measurements by the two methods agreed well. This shows that our method for preparing myofibrils does not affect significantly the sarcomere lengths in the psoas muscle fibers.

Rapid flow quench experiments

The ATPase activity of the shortening myofibrils was measured in a homebuilt, thermostatically controlled, rapid flow quench apparatus (Barman and Travers, 1985). The procedure was to mix myofibrils with [γ-^{32}P]ATP in the apparatus, allowing the mixtures to age (0.3 s and up; see figures), then quenching them in 22% trichloroacetic acid + 1 mM KH_2PO_4, and then to determine the $^{32}P_i$ by the filter paper method of Reimann and Umfleet (1978). The P_i thus determined is the sum of free and enzyme-bound P_i.

In certain experiments the sarcomere lengths of shortening myofibrils were studied by the rapid flow quench method. The myofibrils were mixed with nonradioactive ATP, quenched in 0.15 M K-acetate buffer, pH 4.5, and the sarcomere lengths measured under the optical microscope. At pH 4.5 the shortening and ATPase activity are stopped, and the sarcomere structure of the myofibrils appears to remain intact (Ohno and Kodama, 1991; Ma and Taylor, 1994). In certain experiments, reaction mixtures were quenched in EDTA (3 mM in the basic buffer; see below).

Treatment of flow quench data

A typical myofibrillar ATPase progress curve is illustrated in Fig. 1. It consists of three phases: a P_i burst and two apparently linear phases, k^F and k^S. Our main objective was to obtain estimates for k^F and k^S and the time at which these linear phases intersect, defined by t_B. Because of its long duration k^S was easy to obtain: in Fig. 1 the data for 3 s and later were fitted to a straight line. k^F was of a shorter duration, as it is squeezed between the transient of the initial P_i burst and k^S. However, on the time scale of our experiments and at the ATP concentrations used the transient phase never interfered (Houadjeto et al., 1992), so k^F was obtained by fitting the data from the first time point (0.3 s) up to a straight line. Data points were progressively included until deviation occurred (in Fig. 1 at 2.2 s).

Experimental conditions and chemicals

In all of the experiments the basic buffer was 0.1 M K-acetate, 5 mM KCl, 2 mM Mg-acetate, and 50 mM Tris-acetate, adjusted to pH 7.4 with acetic acid. In the buffer used for the preparation of myofibrils of different sarcomere lengths ("rigor buffer"), the buffer also contained 2 mM EDTA and 1 mM dithiothreitol. In the rapid flow quench experiments, it also contained 0.1 mM $CaCl_2$. Unless otherwise stated, the experiments were carried out at 4°C.

P^1,P^2-Bis(adenosine-5′)pentaphosphate was from Sigma Chemical Co., BDM was from Fluka Chemie Switzerland, and [γ-^{32}P]ATP was from Amersham International.

RESULTS

Measurements of myofibrillar sarcomere lengths

These measurements were essential for our work, in particular for the shortening velocity determinations. Although in a given myofibril the sarcomeres appeared to have identical lengths, to obtain precise lengths with a population of myo-

fibrils is quite difficult. We explain how we set about this, with reference to Fig. 1.

Initial sarcomere lengths

These refer to the sarcomere lengths of myofibrils as prepared, i.e., just before an experiment with the rapid flow quench apparatus. Whether the myofibrils had been prepared by the standard method or from fiber bundles of adjusted sarcomere lengths, the lengths were reasonably homogeneous between myofibrils and between preparations. In a typical standard preparation, the sarcomeres measured 2.65 ± 0.10 μm (40 myofibrils measured).

Sarcomere lengths at t_B

These were obtained by allowing reaction mixtures to age for time t_B, quenching them in the pH 4.5 buffer, and then measuring the sarcomere lengths under the microscope. Two experiments were carried out, with myofibrils of different initial sarcomere lengths. With the standard preparation (see above) the initial value was 2.65 μm and at t_B 2.05 ± 0.20 μm (33 myofibrils). With myofibrils of initial sarcomere lengths 3.20 ± 0.20 (40 myofibrils) the lengths at t_B were identical. We come to two conclusions from these experiments. First, the sarcomere lengths at t_B appear to be independent of the initial lengths. Second, as seen under the microscope, the sarcomere structure of the myofibrils at t_B seems to be preserved.

Sarcomere lengths during shortening

We made attempts to measure shortening velocities by aging reaction mixtures for different times up to t_B, quenching them at pH 4.5, and then measuring the sarcomere lengths. Because of the small shortening distances involved and a certain lack of homogeneity of the sarcomeres, these attempts were unsuccessful. It did not help matters to quench in EDTA. To measure shortening velocities by this direct method, highly precise measurements of the sarcomere lengths are needed (better than ± 2%).

In the myofibrillar ATPase progress curve there is a rapid burst phase of P_i (kinetics not seen in Fig. 1; see Houadjeto et al., 1992, for these). Is there also a rapid shortening phase? We checked on this by quenching young (0.3 s) reaction mixtures at pH 4.5 and measuring the sarcomere lengths. Two experiments were carried out. In the first, the initial sarcomere lengths were 2.67 ± 0.14 μm and at 0.3 s, 2.65 ± 0.10, and in the second they were 3.20 ± 0.19 and 3.18 ± 0.24, respectively (for each estimate 40 myofibrils were measured). Thus, by our methods there was little sign of any rapid initial shortening phase.

Sarcomere lengths at times > t_B

At 20 s after the start of the experiment (Fig. 1), the sarcomere lengths of the myofibrils were 1.1 ± 0.1 μm (19 myofibrils measured). Therefore, shortening continues after t_B but at a much slower rate. At high ATP concentrations and long reaction times (minutes) the myofibrils loose their structures, as seen with the microscope. At t_B, then, the sarcomeres have shortened to their maximum extend under zero load—any further shortening involves passive resistance (Woledge et al., 1985). We did not investigate further this aspect here.

In conclusion: by our methods we were able to obtain reasonable estimates for myofibrillar sarcomere lengths, but they were not precise enough for shortening velocity determinations by the pH 4.5 quench method. Another approach was necessary.

Effects of initial sarcomere lengths on the ATPase parameters

In our attempt at obtaining myofibrillar shortening velocities, the most interesting chemical parameter appears to be t_B. As illustrated in Fig. 1, t_B is the abscissa of the point of intersection of the two linear phases, k^F and k^S. Theoretically, a single progress curve should allow us to calculate directly the shortening velocity, i.e., from the distance shortened (initial sarcomere length − sarcomere length at t_B) and t_B, assuming that the velocity is constant over this sarcomere length range. This is tricky because, as discussed above, the parameters around the transition are difficult to obtain with precision. Furthermore, t_B itself is almost certainly an overestimation for the duration of the rapid shortening phase (which we assume to be manifested by the rapid ATPase rate, k^F), as suggested by a deceleration of k^F at times approaching t_B.

If k^F is the ATPase of myofibrils that are shortening under zero load, then its duration, i.e., t_B, should vary with the initial sarcomere length. As illustrated in Fig. 2, this does seem to be the case; with myofibrils of initial sarcomere length 2.8 and 3.5 μm, t_B was 2.7 and 6.4 s, respectively. The dependence of t_B upon sarcomere length is illustrated in Fig. 3 *A*. With myofibrils of initial sarcomere length 2.7 to 3.5 μm, the dependence appears to be linear,

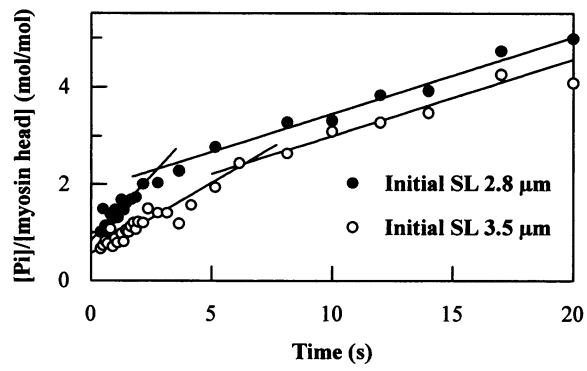

FIGURE 2 Effect of the initial sarcomere length (SL) on the myofibrillar ATPase parameters. The reaction mixtures were 2 μM in myosin heads plus 60 μM [γ-^{32}P]ATP.

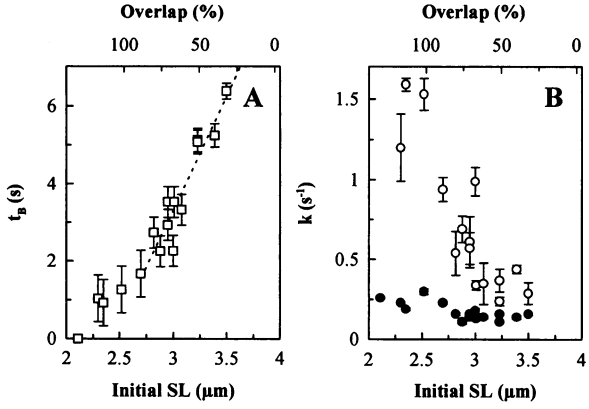

FIGURE 3 Dependences of the ATPase parameters upon the initial sarcomere length (SL). (*A*) t_B and (*B*) k^F (○) and k^S (●). Filament overlap was taken to be 100% at 2.54 μm and 0% at 3.94 μm (Woledge et al., 1985). For definition of the parameters and experimental details, see legends to Figs. 1 and 2.

but when they were less than 2.5 μm, the "breaks" in the ATPase progress curves were difficult to obtain and it was hard to measure t_B and k^F. This is because the shorter the initial sarcomere length, the shorter the duration of k^F (i.e., t_B). When the initial sarcomere lengths were 2.1 μm (i.e., as at t_B), the fast ATPase disappeared altogether and the progress curve was only biphasic: a rapid P_i burst phase was followed by a slow ATPase of 0.26 s^{-1} (result not illustrated). We exploit these results below (Discussion) to obtain estimates of myofibrillar shortening velocities.

The effects of the initial sarcomere lengths on the two ATPases of shortening myofibrils are illustrated in Fig. 3 *B*. As can be seen, as the sarcomere length is increased, k^F decreases but k^S does not change significantly.

It is noteworthy that both the amplitude of the initial P_i burst and the ATP used during the rapid shortening phase (ATP_c) are independent of the initial sarcomere length (Fig. 4). The mean value for the P_i burst amplitude is 0.67 ± 0.12 mol P_i/mol myosin head and for ATP_c, 2.4 ± 0.3 mol P_i/mol head.

Effects of the concentration of ATP on the myofibrillar ATPase parameters

Cooke and Bialek (1979) showed that with muscle fibers, the unloaded shortening velocity is more sensitive to the ATP concentration than their ATPase. Accordingly, we tested the effect of the ATP concentration on the myofibrillar ATPase parameters.

ATPase progress curves at two ATP concentrations are shown in Fig. 5, and it is clear that k^F, k^S, and, in particular, t_B are sensitive to the ATP concentration.

The dependence of k^F, k^S, and t_B upon the ATP concentration is shown in Fig. 6. For the sake of convenience, in the t_B dependence, $1/t_B$ was plotted against ATP concentration. In all the dependences, the hyperbolas fit the data reasonably well.

The K_m values for k^F and k^S are similar (8.6 and 5.3 μM, respectively). The concentration of ATP giving 50% of the maximum value of $1/t_B$ is significantly higher at 60 ± 15 μM. The K_m values are summarized in Table 1, where they are compared with those obtained with muscle fibers.

Effect of ADP and P_i on the myofibrillar ATPase parameters

Cooke and Pate (1985) showed that the isotonic shortening velocity of muscle fibers under zero load is affected in different ways by the products of the ATP hydrolysis; ADP inhibits but P_i has no effect.

All three of the myofibrillar ATPase parameters were sensitive to the presence of ADP; k^F and k^S decreased, whereas t_B increased (results not illustrated). In these experiments, the reaction mixtures contained 25 μM P^1,P^2-bis(adenosine-5′)pentaphosphate. The K_i values obtained are given in Table 1; in all three cases it was assumed that ADP is a competitive inhibitor. The K_i of ADP for k^F is the same as that found from the inhibition of the kinetics of the

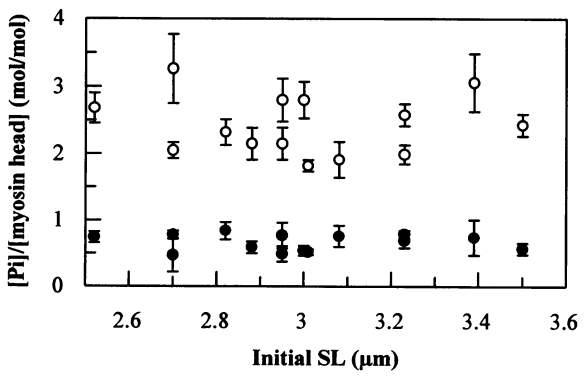

FIGURE 4 Dependences of the amplitude of the P_i burst (●) and ATPc (○) upon the initial sarcomere length (SL). ATP_c is the ATP used per myosin head (mol/mol) at time t_B. For details, see Fig 1. and the legend to Fig. 3.

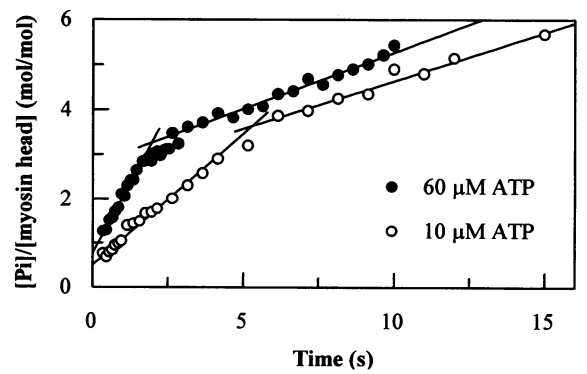

FIGURE 5 Effect of ATP concentration upon the myofibrillar ATPase parameters. The reaction mixtures were (○) 1 μM myosin heads plus 10 μM [γ-^{32}P]ATP and (●) 5 μM myosin heads plus 60 μM [γ-^{32}P]ATP.

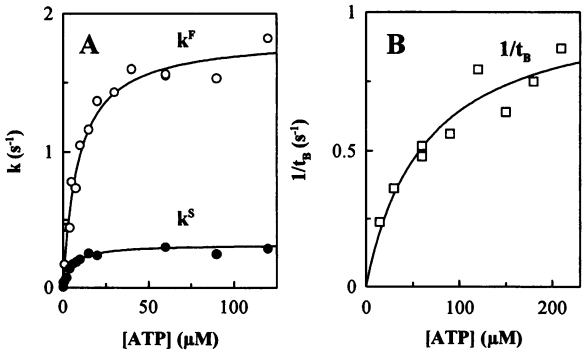

FIGURE 6 Dependences of the ATPase parameters of myofibrils upon the ATP concentration. (*A*) k^F (○) and k^S (●), (*B*) $1/t_B$. To ensure accuracy, the molar ratio of myosin heads to ATP was 1:10 to 1:30. For definition of the parameters and further experimental details, see legends to Figs. 1 and 5.

binding of ATP to rigor-activated myofibrils under identical experimental conditions (Sleep et al., 1994). Johnson and Adams (1984) measured the binding of ADP to rigor myofibrils by a centrifugation method and estimated a K_d of 120 μM (at 0°C). Using a similar method but correcting for the volume of the myofibrils, Biosca et al. (1988) obtained a K_d for ADP of 100 μM (25°C).

ADP also inhibited $1/t_B$. The K_i obtained (150 μM) agrees well with the 200–300 μM estimated by Cooke and Pate (1985) for the isotonic contraction velocity of muscle fibers.

P_i at 5 mM had little effect on k^F, t_B, and k^S. The lack of effect of P_i on t_B is in accord with the findings of Cooke and Pate (1985), who found that 12 mM P_i did not affect the shortening velocity of muscle fibers.

Effect of BDM on the myofibrillar ATPase parameters

An approach to studying mechanochemical coupling in muscle fibers is to seek inhibitors that interact with and stabilize specific intermediates on the ATPase cycle of the cross-bridges. An example of such an inhibitor is BDM, which has been shown to be an uncompetitive inhibitor of myosin ATPase in that it interacts specifically with the M · ADP · P_i state, which is thereby stabilized (Herrmann et al., 1992; McKillop et al., 1994). The effects of BDM on the mechanical parameters of contracting fibers have been studied extensively (e.g., Higuchi and Takemori, 1989; Bagni et al., 1992; Zhao et al., 1995). In particular, BDM reduces the shortening velocity of rabbit psoas muscle fibers (Higuchi and Takemori, 1989).

The three myofibrillar ATPase parameters, k^F, k^S, and t_B, were all affected by BDM. The dependences (not illustrated) were treated on the assumption that BDM is an uncompetitive inhibitor, giving K_i = 2.2 (± 0.4) mM for k^F, 1.1 (± 0.2) mM for k^S, and 5.7 (± 0.4) mM for $1/t_B$. It is noteworthy that the K_i value for the k_{cat} of myosin S1 is about 1 mM (Herrmann et al., 1992).

Temperature dependences of k^F, t_B, and k^S

The three temperature dependences are illustrated in Fig. 7. The similarity of the $\Delta H^{\ddagger}$ values for k^F and k^S (68 ± 2 and 67 ± 3 kJ · mol^{-1}, respectively) suggests that the mechanisms of the two ATPases are similar. We have already discussed the temperature dependence of k^F (Herrmann et al., 1994).

The temperature dependence of t_B gives a $\Delta H^{\ddagger}$ of 44 ± 2 kJ · mol^{-1}, which accords well with a Q_{10} of about 2 for the V_o of muscle fibers (Woledge et al., 1985).

DISCUSSION

Is t_B an indicator of myofibrillar shortening velocities?

As pointed out in the Results, shortening velocities are difficult to obtain by the direct acid quench method, i.e., by optical measurements of sarcomere lengths. Another approach involves determining the dependence of t_B upon the initial sarcomere length. The dependence (Fig. 3 *A*) appears to be linear in the length range 2.7 to 3.5 μm. From this, we can estimate a shortening velocity by making two assumptions. First, we assume that at t_B the sarcomere lengths are independent of the initial sarcomere lengths. We checked on this (see Results). Second, we assume that the overestimation of t_B is also independent of the initial sarcomere length. If the shortening velocity is constant until t_B (with muscle fibers V_o is probably constant during shortening; e.g., Moss, 1982, but also see Brenner, 1980) then

$$V = 1/2(SL_{in} - SL'_{t_B})/(t_B - t_0),$$

where V is the shortening velocity per half-sarcomere (as usually given in physiological studies), t_0 is the overestimate in t_B, and SL_{in} and SL'_{t_B} are the sarcomere lengths initially and at $t_B - t_0$, respectively. Rearranging,

$$t_B = (SL_{in}/2V) - ((SL'_{t_B}/2V) - t_0). \quad (1)$$

Thus, from a plot of t_B versus SL_{in}, we should obtain a straight line of slope $1/2V$, which is independent of SL'_{t_B} and t_0. From the linear portion of the plot in Fig. 3 *A*, V = 0.085 ± 0.010 μm · s^{-1}/half-sarcomere.

Equation 1 predicts that with myofibrils containing sarcomeres of a given length, $1/t_B$ is a function of V. Therefore, to test further $1/t_B$ as a probe of V, we determined its dependence on the ATP concentration and the effects of the addition of ADP, P_i, or BDM. As summarized in Table 1, the effects are similar to those found with muscle fibers. It is noteworthy that the concentration of ATP that gives half the maximum effect is significantly higher for $1/t_B$ than for k^F, as found for the V_o and ATPase of muscle fibers shortening under zero external load (Cooke and Bialek, 1979).

How do myofibrillar shortening velocities compare with those of fibers? Myofibrillar shortening velocities have been measured directly under the optical microscope (Harada et al., 1990; Harrington et al., 1990). In those experiments,

TABLE 1 Probing the ATPase parameters of myofibrils* and the shortening velocity of muscle fibers

Probe	Myofibrils (4°C)			Fibers (10°C)	
	k^F	k^S	t_B	V_o	References
K_m of ATP (μM)	8.6 ± 1.1	5.3 ± 0.8	>60 ± 15	150	Cooke and Bialek, 1979
K_i of ADP (μM)	150 ± 18	72 ± 12	240 ± 20	200–300	Cooke and Pate, 1985
P_i		Little effect at 5 mM		Little effect at 12 mM	Cooke and Pate, 1985
K_i of BDM (mM)	2.2 ± 0.4	1.1 ± 0.2	5.7 ± 0.4	Inhibition	Higuchi and Takemori, 1989, and refs. cited therein
$\Delta H^{\ddagger}$ (kJ · mol^{-1})	68 ± 2	67 ± 3	44 ± 2	About 50	Woledge et al., 1985

*The ATPase parameters of contracting myofibrils are defined in Fig. 1.

myofibrils were mixed with caged ATP, and shortening was triggered by a laser flash. It is difficult to compare these measurements with ours, because in the former any remaining caged ATP was not taken account of; caged ATP inhibits ATP binding to myofibrils and, therefore, shortening velocities (Sleep et al., 1994). Here we obtained a shortening velocity of 0.085 μm · s^{-1}/half-sarcomere at 4°C and at an ATP concentration of 60 μM. The concentration giving a half-maximum value of $1/t_B$ determined from Fig. 6 *B* is 60 μM, giving a maximum value V_o of 0.17 μm · s^{-1}/half-sarcomere. However, as t_B is an overestimation of the duration of the rapid shortening, 60 μM is an underestimation. Working with skinned psoas fibers, Cooke and Bialek (1979) obtained directly the dependence of shortening velocity (at zero external load) on the ATP concentration and estimated a K_m of 150 μM. With this K_m, we obtain a V_o for our myofibrils of about 0.3 μm · s^{-1}/half-sarcomere at 4°C. This is reasonabley close to the the 0.57 value obtained for skinned psoas fibers at 2°C (Brenner, 1980), but, of course, myofibrils and fibers may not have identical shortening velocities.

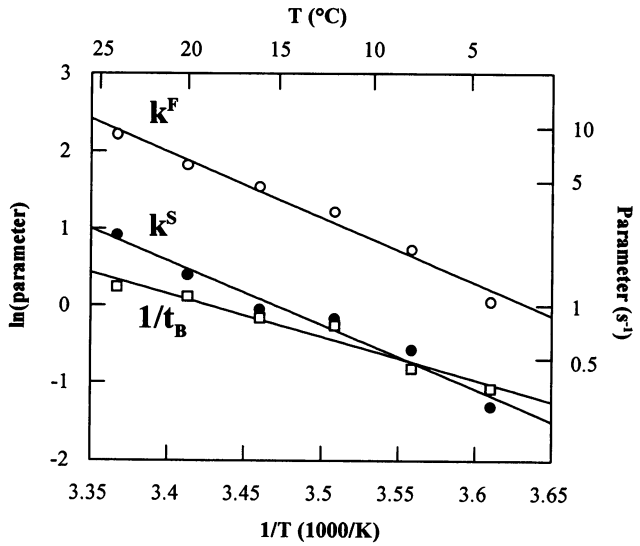

FIGURE 7 Arrhenius plots of the myofibrillar ATPase parameters k^F, k^S, and $1/t_B$. For definitions and experimental details, see Fig. 1.

We conclude from these experiments that it is reasonable to suppose that t_B can be used as an indicator of the shortening velocity of myofibrils.

Effect of initial sarcomere length on myofibrillar ATPase kinetics

As discussed above, the effect of increasing the initial sarcomere length is to increase t_B, but it also decreases k^F, the ATPase of the rapidly shortening myofibrils (Fig. 3 *B*).

The ATPase parameters are not independent, as they are connected by

$$k^F = (ATP_c - P_i\text{burst size})/t_B.$$

From our experiments, it appears that ATP_c and the P_i burst size are independent of the initial sarcomere length (Fig. 4). Therefore, the product $k^F \cdot t_B$ is also independent. We schematize this situation in Fig. 8. How to interpret? From a purely enzymatic point of view, the situation is difficult to understand; we have made several attempts at fitting it to classical ATPase schemes, but without success. The main difficulties are as follows. First, during the course of the experiment, the ATPase sites change continuously because the filament overlap increases, so more and more myosin heads may come in contact with actin. Second, during the

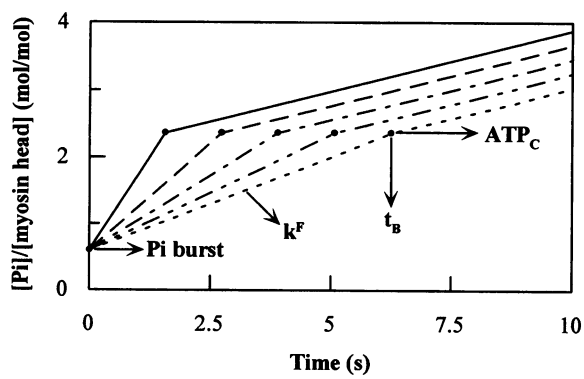

FIGURE 8 Schematic representation of the dependence of the myofibrillar ATPase parameters upon the initial sarcomere length. The curves were drawn from the dependences in Figs. 3 and 4 with initial sarcomere lengths (left to right) 2.7, 2.9, 3.1, 3.3, and 3.5 μm.

rapid shortening phase, the amount of P_i produced per myosin head is low (about 2 mol P_i). Does this phase involve all of the myosin heads, in which case the conditions would be nearly single turnover, or only a few heads under clearly multiturnover conditions? From our results we cannot tell. Third, the observed kinetics, k^F, were reasonably linear, whatever the initial sarcomere length. In particular, no acceleration phase was discerned in any of the experiments. Of course, small acceleration phases cannot be excluded, but we feel that our analytical methods are good enough to detect these when significant. The lack of acceleration phases is surprising; with myofibrils of long sarcomere length presumably few heads are initially attached, but as the shortening proceeds, their number increases. Therefore, one would expect k^F to increase as shortening proceeds. It is possible that this phenomenon is due to the myosin heads needing a certain time to acquire their actin-induced conformation (i.e., high ATPase) upon attachment to the thin filament. If this time is longer than the k^F measurement, k^F would be constant, even at low starting overlap. The possibility that the myosin heads go through slow actin-related changes has been discussed (Biosca et al., 1984; Lombardi et al., 1992).

To conclude, we have mechanical and chemical kinetic data on shortening myofibrils that we are unable to fit to a kinetic scheme. But we can discuss these data with reference to muscle fiber work.

Myofibrillar and muscle fiber ATPases

The effect of an increase in sarcomere length in decreasing k^F, the ATPase of the rapidly shortening myofibrils, is in agreement with data from muscle fiber work. Hayashi and Tonomura (1968) were the first to show that the ATPase activity of fibers decreases with sarcomere length. Stephenson et al. (1989) studied the effect of the sarcomere length on the Ca-activated activity of isometrically held rat muscle fibers (extensor digitorum longus muscle). At 100% overlap (sarcomere length about 2.5 μm) the ATPase was about 4 s^{-1} (at 22°C), whereas at 4 μm (zero overlap) the activity was only about 0.4 s^{-1}. The decrease in ATPase with excessive increase in sarcomere length is reasonable, as it is presumably only the attached cross-bridges that contribute significantly to the overall ATPase. Thus, as the sarcomere length increases, fewer and fewer cross-bridges remain attached to the thin filament (i.e., overlap decreases) and the ATPase is reduced.

With myofibrils, the slow ATPase, k^S, was insensitive to sarcomere length (Fig. 3 *B*). This makes sense, as k^S is the ATPase of the myofibrils after t_B, i.e., after their rapid shortening phase. At t_B, the percentage overlap seems to be independent of the initial sarcomere length.

It is noteworthy that the amount of ATP used during shortening appears to be independent of the distance moved. This implies that the more the myofibrils shorten, the less the ATP used per head per micron shortened. This economy of ATP is in accord with the findings of Higuchi and Goldman (1991, 1995) and Lombardi et al. (1992), who proposed that in the shortening muscle fiber the free energy of one ATP hydrolyzed is used in several actin-myosin interactions. It is also in accord with the weakly coupled model of Cooke et al. (1994).

These considerations on the economy of ATP lead directly to the problem of the sliding distance, i.e., the physical distance traveled by a cross-bridge along the thin filament per molecule of ATP hydrolyzed. The classical cross-bridge theory (e.g., Huxley, 1980) assumes that this is less than 40 nm, a value limited by the physical dimensions of the myosin head. As discussed by Finer et al. (1994), the sliding distance is a problem because its measurement is based on a number of assumptions, in particular on the number of myosin heads involved.

It is difficult to come to any conclusions as to the sliding distance with myofibrils. From our experiments, the sliding distance seems to increase with the initial sarcomere length. Thus, if we assume that the rapid shortening stops when the sarcomere length is 2.05 μm (at t_B), then with myofibrils of initial sarcomere length 2.7 μm, the sliding distance is 0.65 μm/2.4 mol ATP/mol myosin head (mean value, Fig. 4), i.e., 270 nm. When the initial sarcomere length is 3.5 μm, the sliding distance is 600 nm. This increase in sliding distance with initial sarcomere length fits in with muscle fiber work: fiber ATPase decreases with sarcomere length (Stephenson et al., 1989), whereas V_o is independent (Edman, 1979). Very recently, Higuchi and Goldman (1995) reported long sliding distances during isotonic shortening of skinned muscle fibers. We point out that our estimates with myofibrils are based upon the assumption that all of the myosin heads participate in the shortening process, which may not be the case. Clearly, if only a few heads are involved, with the remaining heads as passengers (Irving, 1991), then the sliding distance would be shorter. Further experiments are needed to resolve this problem.

FINAL COMMENTS

Here we propose that a chemical kinetic parameter obtained from myofibrillar ATPase progress curves, t_B, is a manifestation of their shortening velocity. Thus, we propose that in the same experiment we obtain mechanical and chemical kinetic data. Does this help understanding of myofibrillar mechanochemical coupling?

The rate-limiting step on the myofibrillar ATPase reaction pathways is the P_i release (Lionne et al., 1995a), which suggests strongly that the predominant intermediates contain the products, of the type $AM \cdot ADP \cdot P_i$ and $M \cdot ADP \cdot P_i$ (where A represents actin and M a myosin head). Now, these intermediates are formed very rapidly (Houadjeto et al., 1992) compared with the duration of the shortening phase (i.e., t_B). Therefore, for almost the totality of the shortening process, the majority of the heads are in the $ADP \cdot P_i$ states, which are then the key intermediates in

both the kinetics of P_i release and the shortening velocity. This would explain the sensitivity of both to BDM, which interacts specifically with the ADP · P_i states (Herrmann et al., 1992; McKillop et al., 1994). Predominant ADP · P_i states would also support the suggestion of Homsher (1987) that during the rapid shortening of muscle fibers, myosin heads are "torn off" the thin filament: it would be easier to pull the heads off the "weak" actin-binding states AM · ADP · P_i than strong states such as AM or AM · ADP.

Finally, we must emphasize the richness of the myofibrillar ATPase progress curves. They provide estimates of five myofibrillar shortening parameters: the concentration of the product state AM·ADP·P_i (P_i burst size), the ATPase of shortening myofibrils (k^F), the amount of ATP expended during the shortening (ATP_c), an indication of the shortening velocity (t_B), and the ATPase of overcontracted myofibrils (k^S). It has not escaped our notice that these curves could serve as a test for the effect of various substances, especially drugs, on the myofibrillar shortening parameters.

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REFERENCES

Bagni, M. A., G. Cecchi, F. Colomo, and P. Garzella. 1992. Effect of 2,3-butanedione monoxime on the crossbridge kinetics in frog single muscle fibres. *J. Muscle Res. Cell Motil.* 13:516–522.

Barman, T. E., and F. Travers. 1985. The rapid-flow-quench method in the study of fast reactions in biochemistry: extension to subzero conditions. *Methods Biochem. Anal.* 31:1–59.

Bartoo, M. L., V. I. Popov, L. A. Fearn, and G. H. Pollack. 1993. Active tension generation in isolated skeletal myofibrils. *J. Muscle Res. Cell Motil.* 14:498–510.

Biosca, J. A., T. E. Barman, and F. Travers. 1984. Transient kinetics of the binding of ATP to actomyosin subfragment 1: evidence that the dissociation of actomyosin subfragment 1 by ATP leads to a new conformation of subfragment 1. *Biochemistry.* 23:2428–2436.

Biosca, J. A., L. E. Greene, and E. Eisenberg. 1988. Binding of ADP and 5′-adenylyl imidodiphosphate to rabbit muscle myofibrils. *J. Biol. Chem.* 263:14231–14235.

Brenner, B. 1980. Effect of free sarcoplasmic Ca^{2+} concentration on maximum unloaded shortening velocity: measurements on single glycerinated rabbit psoas muscle fibres. *J. Muscle Res. Cell Motil.* 1:409–428.

Brune, M., J. L. Hunter, J. E. T. Corrie, and M. R. Webb. 1994. Direct, real-time measurement of rapid inorganic phosphate release using a novel fluorescent probe and its application to actomyosin subfragment 1 ATPase. *Biochemistry.* 33:8262–8271.

Colomo, F., N. Piroddi, C. Poggesi, and C. Tesi. 1995. Myofibrillar contribution to passive tension in striated muscle. *Biophys. J.* 68:344S.

Cooke, R., and W. Bialek. 1979. Contraction of glycerinated muscle fibers as a function of the ATP concentration. *Biophys. J.* 28:241–258.

Cooke, R., and E. Pate. 1985. The effects of ADP and phosphate on the contraction of muscle fibers. *Biophys. J.* 48:789–798.

Cooke, R., H. White, and E. Pate. 1994. A model of the release of myosin heads from actin in rapidly contracting muscle fibers. *Biophys. J.* 66: 778–788.

Edman, K. A. P. 1979. The velocity of unloaded shortening and its relation to sarcomere length and isometric force in vertebrate muscle fibres. *J. Physiol.* 291:143–159.

Finer, J. T., R. M. Simmons, and J. A. Spudich. 1994. Single myosin molecule mechanics: piconewton forces and nanometre steps. *Nature.* 368:113–119.

Friedman, A. L., and Y. E. Goldman. 1993. Force and force transients in bundles of 1–3 myofibrils from rabbit psoas muscle. *Biophys. J.* 64:A345.

Friedman, A. L., and Y. E. Goldman. 1994. Active ramp shortening of bundles of 1–3 myofibrils from rabbit psoas muscle. *Biophys. J.* 66:A5.

Goldman, Y. E. 1987. Kinetics of the actomyosin ATPase in muscle fibers. *Annu. Rev. Physiol.* 49:637–654.

Harada, Y., K. Sakurada, T. Aoki, D. D. Thomas, and T. Yanagida. 1990. Mechanochemical coupling in actomyosin energy transduction studied by in vitro movement assay. *J. Mol. Biol.* 216:49–68.

Harrington, W. F., T. Karr, W. B. Busa, and S. J. Lovell. 1990. Contraction of myofibrils in the presence of antibodies to myosin subfragment 2. *Proc. Natl. Acad. Sci. USA.* 87:7453–7456.

Hayashi, Y., and Y. Tonomura. 1968. Dependence of activity of myofibrillar ATPase on sarcomere length and calcium ion concentration. *J. Biochem.* 63:101–118.

Herrmann, C., C. Lionne, F. Travers, and T. Barman. 1994. Correlation of actoS1, myofibrillar, and muscle fiber ATPases. *Biochemistry.* 33: 4148–4154.

Herrmann, C., J. Sleep, P. Chaussepied, F. Travers, and T. Barman. 1993. A structural and kinetic study on myofibrils prevented from shortening by chemical cross-linking. *Biochemistry.* 32:7255–7263.

Herrmann, C., J. Wray, F. Travers, and T. Barman. 1992. Effect of 2,3-butanedione monoxime on myosin and myofibrillar ATPases. An example of an uncompetitive inhibitor. *Biochemistry.* 31:12227–12232.

Higuchi, H., and Y. E. Goldman. 1991. Sliding distance between actin and myosin filaments per ATP molecule hydrolysed in skinned muscle fibres. *Nature.* 352:352–354.

Higuchi, H., and Y. E. Goldman. 1995. Sliding distance per ATP hydrolyzed by myosin heads during isotonic shortening of skinned muscle fibres. *Biophys. J.* 69:1491–1507.

Higuchi, H., and S. Takemori. 1989. Butanedione monoxime suppresses contraction and ATPase activity of rabbit skeletal muscle. *J. Biochem.* 105:638–643.

Homsher, E. 1987. Muscle enthalpy production and its relationship to actomyosin ATPase. *Annu. Rev. Physiol.* 49:673–690.

Houadjeto, M., T. Barman, and F. Travers. 1991. What is the true ATPase activity of contracting myofibrils? *FEBS Lett.* 281:105–107.

Houadjeto, M., F. Travers, and T. Barman. 1992. Ca^{2+}-activated myofibrillar ATPase: transient kinetics and the titration of its active sites. *Biochemistry.* 31:1564–1569.

Huxley, A. F. 1980. Reflections on Muscle. Liverpool University Press, Liverpool.

Irving, M. 1991. Biomechanics goes quantum. *Nature.* 352:284–285.

Johnson, R. E., and P. H. Adams. 1984. ADP binds similarly to rigor muscle myofibrils and to actomyosin-subfragment one. *FEBS Lett.* 174: 11–14.

Knight, P. J., and J. A. Trinick. 1982. Preparation of myofibrils. *Methods Enzymol.* 85B:9–12.

Lionne, C., M. Brune, M. R. Webb, F. Travers, and T. Barman. 1995a. Time resolved measurements show that phosphate release is the rate limiting step on myofibrillar ATPases. *FEBS Lett.* 364:59–62.

Lionne, C., C. Herrmann, F. Travers, and T. Barman. 1995b. The myofibril as a model for muscle fibre ATPase. *Biophys. J.* 68:217s.

Lombardi, V., G. Piazzesi, and M. Linari. 1992. Rapid regeneration of the actin-myosin power stroke in contracting muscle. *Nature.* 355:638–641.

Ma, Y.-Z., and E. W. Taylor. 1994. Kinetic mechanism of myofibril ATPase. *Biophys. J.* 66:1542–1553.

McKillop, D. F. A., N. S. Fortune, K. W. Ranatunga, and M. A. Geeves. 1994. The influence of 2,3-butanedione 2-monoxime (BDM) on the interaction between actin and myosin in solution and in skinned muscle fibres. *J. Muscle Res. Cell Motil.* 15:309–318.

Moss, R. L. 1982. The effect of calcium on the maximum velocity of shortening in skinned skeletal muscle fibres of the rabbit. *J. Muscle Res. Cell Motil.* 3:295–311.

Ohno, T., and T. Kodama. 1991. Kinetics of adenosine triphosphate hydrolysis by shortening myofibrils from rabbit psoas muscle. *J. Physiol.* 441:685–702.

Reimann, E. M., and R. A. Umfleet. 1978. Selective precipitation of $^{32}P_i$ onto filter papers. *Biochim. Biophys. Acta.* 523:516–521.

Sleep, J., C. Herrmann, T. Barman, and F. Travers. 1994. Inhibition of ATP binding to myofibrils and acto-myosin subfragment 1 by caged ATP. *Biochemistry.* 33:6038–6042.

Stephenson, D. G., A. W. Stewart, and G. J. Wilson. 1989. Dissociation of force from myofibrillar MgATPase and stiffness at short sarcomere lengths in rat and toad skeletal muscle. *J. Physiol.* 410:351–366.

Woledge, R. C., N. A. Curtin, and E. Homsher. 1985. Energetic aspects of muscle contraction. *Monogr. Physiol. Soc.* 41:1–357.

Zhao, L., N. Naber, and R. Cooke. 1995. Muscle cross-bridges bound to actin are disordered in the presence of 2,3-butanedione monoxime. *Biophys. J.* 68:1980–1990.