Ca^{2+}-Activated Myofibrillar ATPase: Transient Kinetics and the Titration of Its Active Sites†

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ABSTRACT: The transient kinetics of rabbit psoas Ca^{2+}-activated myofibrillar Mg^{2+}-ATPase were studied in a buffer of near physiological ionic strength at 4 °C by the rapid flow quench technique. The initial ATP binding steps were studied by the ATP chase and the cleavage and release of products steps were studied by the P_i burst method. The data obtained were interpreted by the simple scheme shown below, where M

$$M + ATP \underset{}{\overset{K_1}{\rightleftharpoons}} M{\cdot}ATP \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} M^*{\cdot}ATP \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} M^{**}{\cdot}ADP{\cdot}P_i \xrightarrow{k_4} M + ADP + P_i$$

represents the myosin heads with or without actin interaction. The constants obtained with myofibrils (where the molecules are highly organized) were compared with those with myosin subfragment 1 (S1) and cross-linked acto-S1 (where the molecules are dispersed in solution). Myofibrils appear to bind ATP as tightly as do S1 and cross-linked acto-S1. This suggests that with them $k_{-2} < k_{cat} \ll k_2$, and it is proposed that the ATP chase method can be used to titrate the ATPase sites in myofibrils. The results of titration and single-turnover experiments revealed that myofibrils may contain partially active myosin heads. It is proposed that these heads bind ATP loosely without hydrolysis, as found with S1 [Tesi, C., N. Bachouchi, N., Barman, T., & Travers, F. (1989) *Biochimie 71*, 363–372]. There were large P_i bursts with the three preparations, showing that with all of them the release of products step (k_4) is rate limiting. Remarkably, the second-order binding constant for ATP (k_2/K_1, 1 $\mu M{\cdot}s^{-1}$) and the kinetics of the cleavage step ($k_3 + k_{-3}$, about 20 s^{-1}) were similar for the three materials, but a subtle difference in the cleavage step of myofibrillar ATPase cannot be excluded. The release of products step (k_4) was similar with myofibrils and cross-linked acto-S1 (about 2 s^{-1}) but much faster than with S1 (0.02 s^{-1}).

Myosin is the major component of the thick filament of the muscle fiber, and at its globular heads ATP[1] is hydrolyzed; it is this hydrolysis that provides the energy for muscle contraction. Muscle contraction depends on the cyclic interaction of the myosin heads (cross-bridges) with actin (the major component of the thin filament). In the classical cross-bridge model [e.g., Huxley (1988)] this interaction is taken to be synchronized by the different intermediates on myosin ATPase.

There is a large amount of data on the kinetics of isolated myosin and reconstituted actomyosin ATPases [e.g., Trentham et al. (1976), Taylor (1979), Adelstein and Eisenberg (1980), Hibberd and Trentham (1986), and Geeves (1991)]. This was obtained under a variety of experimental conditions, mostly in low ionic strength buffers. These works have led toward a fairly detailed knowledge of the physicochemical properties of the interactions in the actin–myosin–ATP system, indispensable toward a full understanding of muscle contraction. However, this knowledge was obtained from studies on molecules dispersed in solution, and any attempt at extrapolating from it to an organized system such as muscle fibers must be done cautiously and selectively.

It is very difficult to study directly the kinetic processes of ATPase in organized systems such as muscle fibers. An elegant way of attacking this problem is to use caged compounds [e.g., Hibberd and Trentham (1986) and Homsher and Millar (1990)], and this novel technique shows great promise.

Another approach for studying the chemical kinetics of muscle contraction is to use myofibrils. Myofibrils are bundled into fibers which in turn are bundled into muscle. They are the functional contractional units of the muscle, and yet they are small enough to be amenable for study in rapid reaction equipment. However, myofibrils are not anchored, and they do not carry out significant work when contracting. As pointed out by Huxley (1980), the rates of at least some of the chemical reactions that occur in the contracting muscle appear to be influenced by mechanical conditions such as load or change of length. Nevertheless, a detailed knowledge of the chemical kinetics of contracting myofibrils is a step toward a full understanding of the mechanics of muscle contraction. Further, studies on myofibrils provide a bridge between studies on dispersed molecules (e.g., acto-S1) and muscle fibers.

There is little data available on the detailed kinetics of activated myofibrils. To prevent overcontraction, Sleep (1981) experimented under single-turnover conditions and concluded that at 0 °C $k_{cat} < 0.45$ s^{-1}. Taylor (1990) maintains that at at 20 °C the P_i burst is small, i.e., that the release of P_i step is not rate limiting.

Harada et al. (1990) studied the contraction and ATPase rates of activated myofibrils at 22 °C. The initial rapid contraction, occurring under near zero-load conditions, lasted for 100 ms, and during it the ATPase activity was 9 s^{-1}. This was followed by a drop in activity to 1.5 s^{-1}, which Harada et al. (1990) suggest is that during isometric contraction. Houadjeto et al. (1991) obtained the time course for Ca^{2+}-activated myofibrillar ATPase at 4 °C. There were three distinct phases: a large P_i burst, a rapid steady-state rate (about 1 s^{-1}), and then, at 1 s, a sharp decrease in activity to

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[1] Abbreviations: ATP, adenosine 5′-triphosphate; P_i, inorganic orthophosphate; S1, myosin subfragment 1; Tris, tris(hydroxymethyl)-aminomethane; DTT, dithiothreitol.

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0.15 s^{-1}. This low activity lasted for at least several minutes. Contracting myofibrils are therefore fleeting structures, and for their kinetic study rapid reaction equipment is needed. Here we extend our preliminary studies to a more detailed transient kinetic investigation of activated myofibrils at 4 °C.

In studies on actomyosin, the choice of the solvent, in particular, ionic strength, is critical. In solution most of the actomyosin interactions are highly susceptible to the ionic strength, and most studies have been carried out at low, nonphysiological ionic strengths. With the organized myofibrils, this is less of a problem, and here we used a solvent of ionic composition near to that obtained in vivo. For the cation, we used K^+ at 0.1 M, which is about that found in the sarcoplasm of muscle fibers (Maughan & Godt, 1989). In vivo, the principal anion is creatine phosphate (Maughan & Godt, 1989), but for technical and cost reasons we used 0.1 M acetate. The pH of the buffer was 7.4. Because of the rapidity of the kinetics, we were unable to experiment at or even near the body temperature of the rabbit (39.1 °C), and we carried out our work at 4 °C.

Our study was confined to chemical sampling using the rapid flow quench method. The transient kinetics of the binding of ATP were studied by the unlabeled ATP chase method (Trentham et al., 1976; Barman & Travers, 1985) and the formation of P_i (enzyme bound and free) by the P_i burst experiments (Taylor, 1979). The data obtained were interpreted according to Scheme I. This is a shortened version of the seven-step Bagshaw–Trentham pathway obtained with myosin alone [e.g., Trentham (1976)]. In it the constant k_4 refers to the release of both products rather than to P_i alone as in the full scheme.

Scheme I

$$M + ATP \underset{}{\overset{K_1}{\rightleftharpoons}} M\cdot ATP \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} M^*\cdot ATP \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} M^{**}\cdot ADP\cdot P_i \xrightarrow{k_4} M + ADP + P_i$$

M represents the myosin heads in myofibrils with or without actin interaction. The scheme does not take into account the interactions of the heads with actin; for want of a suitable method, we did not study these here.

We find that, as with myosin, k_{-2} is small with respect to k_4, which made possible the titration of ATPase sites and an evaluation of the second-order constant k_2/K_1. In P_i burst experiments, there were transient burst phases followed by steady-state rates which made possible estimates of k_3, k_{-3}, and k_4. The kinetics thus obtained were compared with those with S1 and cross-linked acto-S1 (Mornet et al., 1981) under the same conditions.

Materials and Methods

Proteins and Reagents. Myofibrils were prepared from rabbit psoas muscle as in Houadjeto et al. (1991), and myosin, S1, actin, and cross-linked acto-S1 were prepared as in Biosca et al. (1984, 1985). [γ-^{32}P]ATP was from Amersham International.

Analytical Methods. The concentration of protein in myofibrils was determined by dissolving them in 2% sodium dodecyl sulfate and measuring the absorbance at 280 nm using $E_{280}^{1\%} = 7$ (Sutoh & Harrington, 1977). The concentration of myosin heads was calculated assuming that 43% of the myofibrillar protein is myosin (Yates & Greaser, 1983).

Experimental Conditions. Unless otherwise stated, all kinetic experiments were carried out at 4 °C in 0.1 M potassium acetate/5 mM KCl/2 mM magnesium acetate/0.1 mM $CaCl_2$/50 mM Tris adjusted to pH 7.4 with acetic acid.

Rapid Flow Quench Experiments. Two types of experiments were carried out.

ATP chase experiments: A key feature of myosin ATPase is that the binding of ATP is essentially irreversible [i.e., $k_{-2} \ll k_2$ (Scheme I); Mannherz et al., 1974]. Further, the binding is very rapid ($k_2 \gg k_{cat}$). This makes it possible to carry out ATP chase experiments with myosin. In this, myosin plus [γ-^{32}P]ATP reaction mixtures are aged for time t and then mixed in unlabeled ATP. This reaction mixture is then incubated on ice for several turnovers and finally quenched in 22% trichloroacetic acid, and the amount of [^{32}P]P_i is determined. As discussed below, this measured P_i represents tightly bound ATP plus hydrolyzed ATP.

Upon the addition of the cold ATP, the free radioactive ATP and that in the collision complex M·ATP are rapidly diluted out but we assume that in M*·ATP the ATP can only be released by being hydrolyzed to [^{32}P]P_i via k_3 and k_4. With S1, therefore, ATP chase experiments give a rapid and large rise of [^{32}P]P_i.

The complete [^{32}P]P_i progress curve is defined as

$$[[^{32}P]P_i]/[M]_o = (k_{cat}/k_{cat} + k_{-2})(1 - e^{-kt}) + k_{cat}t \quad (1)$$

where $[M]_o$ is the myosin head concentration. Now, since with myosin $k_{-2} \gg k_{cat} \gg k_2$, the amplitude of the transient phase (i.e., [^{32}P]P_i at $t \gg 1/k$) is equal to the ATPase site concentration. The importance of the ATPase chase method is that by its use one can titrate the ATPase sites in myosin. The kinetics of the rise is given by

$$k = k_2[ATP]/([ATP] + K_1) \quad (2)$$

The following steady state is given by $k_{cat}t$ [for a further discussion see Barman and Travers (1985) and Tesi et al. (1989)].

Here we carried out ATP chase experiments with myofibrils and we interpreted the results obtained by the treatment above for myosin.

In *P_i burst experiments*, reaction mixtures are quenched directly in acid and the amount of [^{32}P]P_i is determined. Here one obtains information about the kinetics of the cleavage and release of P_i steps (k_3, k_{-3}, and k_4; Scheme I).

Both types of experiments were carried out in thermostatically controlled rapid flow quench apparatuses constructed in this laboratory (Barman & Travers, 1985). In early experiments with myofibrils, clogging of the apparatus mixers occurred. This was prevented by further homogenization of the myofibrils using a Sorvall Omnimixer. The myofibrils thus obtained contained 5–12 sarcomers.

Treatment of Data. In a given experiment, the data points are [^{32}P]P_i as a function of time. The dependencies of this time course upon the protocol used were determined. In the interpretation of this data, we used the now classical strategy, namely adjusting the kinetic constants describing the simplest plausible scheme to the data points. Here we used an Apple IIe computer with laboratory-made programs based on the integrated forms of the several rate expressions defining Scheme I.

We first interpreted the data points obtained at high ATP concentrations (75 and 100 μM), as under these conditions the ATP chase and P_i burst kinetics are very different. This permitted a first evaluation of the constants. These were then further adjusted to the data points obtained at lower ATP concentrations. Thus, the final set of constants obtained (Table I) is the consensus of Scheme I and the data points from a number of experiments at different ATP concentrations and quenchers.

Table I: Certain Kinetic Constants for Myofibrillar and S1 ATPases at 4 °C[a]

constant	S1	myofibrils (Ca^{2+})
k_2/K_1 ($M^{-1}\cdot s^{-1}$)	1 (±0.2) × 10^6	1 (±0.2) × 10^6
k_3 (s^{-1})	12 (±2)	17 (±2)
k_{-3} (s^{-1})	4 (±1)	1 (±0.5)
$k_3 + k_{-3}$ (s^{-1})	16	18
K_3	3 (±1)	17 (±3)
k_4 (s^{-1})	0.03 (±0.005)	2 (±0.2)
k_{cat} (s^{-1})	0.02 (±0.002)	1.7 (±0.2)

[a] The buffer was 0.1 M potassium acetate/5 mM KCl/2 mM magnesium acetate/50 mM Tris adjusted to pH 7.4 with acetic acid.

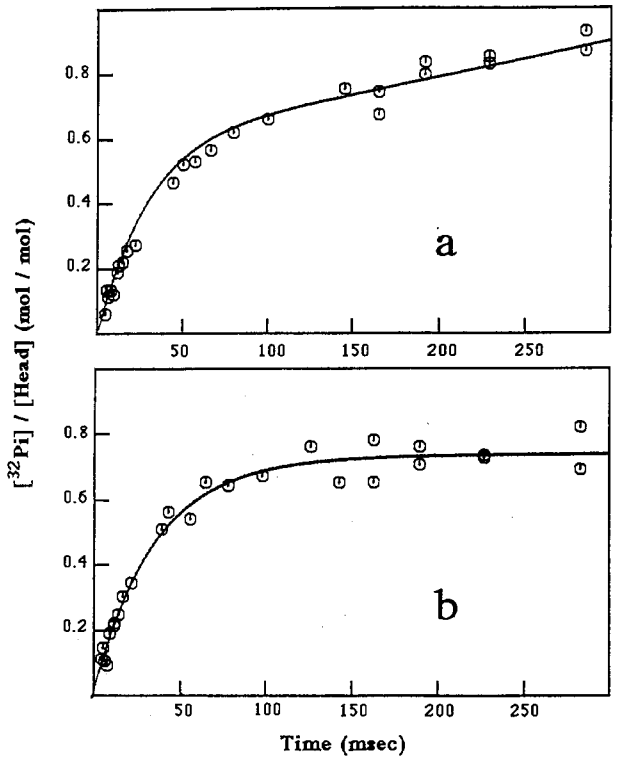

FIGURE 1: Time courses for the binding of ATP to myofibrils (a) and S1 (b). The reaction mixtures were 3 μM myosin heads + 32 μM [γ-^{32}P]ATP (for myofibrils) and 2.7 μM S1 + 28.7 μM [γ-^{32}P]ATP (for S1). They were quenched in 50 mM cold ATP at the times shown, and the amount of [^{32}P]P_i was determined. The buffer was 50 mM Tris–acetate, pH 7.4/0.1 M potassium acetate/5 mM KCl/2 mM magnesium acetate/0.1 mM $CaCl_2$, and the temperature was 4 °C.

RESULTS

ATP Chase Experiments with S1. A typical chase experiment with S1 is given in Figure 1b at 28.7 μM ATP: there was a rapid rise of tightly bound ATP (kinetics, k = 28.8 s^{-1}; amplitude, 0.73 mol of ATP bound/mol of S1). The rapid rise was followed by a slow steady-state phase (0.02 s^{-1}, not shown). In the concentration range used, the dependence of k upon ATP concentration was linear and it was not possible to determine saturation kinetics under the experimental conditions used (data not shown). K_1 and k_2 (eq 2) are therefore large; from the slope of the curve was obtained the second-order binding constant k_2/K_1 (Table I).

ATP Chase Experiments with Myofibrils and Cross-Linked Acto-S1. A typical ATP chase experiment with myofibrils is illustrated in Figure 1a. This was carried out under conditions very similar to those in the experiments with S1 (Figure 1b). As for S1, there was a rapid rise of bound ATP which was followed by a rapid steady-state rate (1.7 s^{-1}), confirmed by an experiment in the hundreds of milliseconds time range [not shown, but see Houadjeto et al. (1991)]. Since with myofibrils k_{cat} is fast, the interpretation of chase experiments

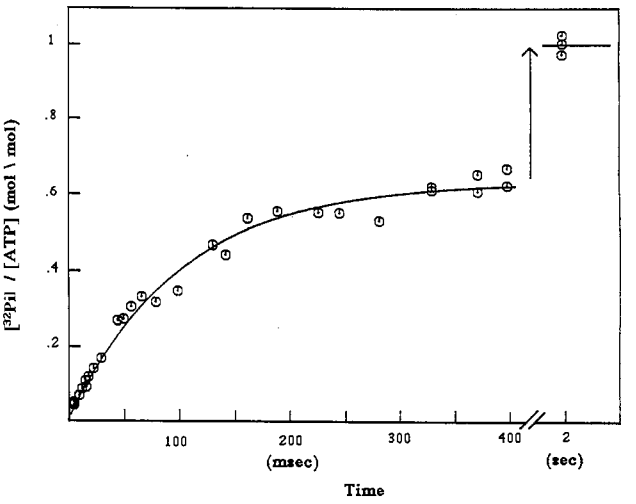

FIGURE 2: ATP chase experiment with myofibrils under single-turnover conditions. The reaction mixtures (13 μM myosin heads + 0.6 μM [γ-^{32}P]ATP) were quenched in 50 mM cold ATP, and the amount of [^{32}P]P_i was determined. The curve was fitted to a single exponential (k = 9.9 s^{-1}) with an amplitude of 0.63 mol of [^{32}P]P_i/mol of [γ-^{32}P]ATP. For experimental conditions, see legend to Figure 1.

with them is not as easy as that with of S1, but as a first approximation the curves obtained were well fitted by eq 1 and the values for k obtained (31 s^{-1} under the conditions of Figure 1a). Further, the dependence of k upon the ATP concentration appears to be linear and very similar to that with S1, and the data could be fitted to a second-order constant of 10^6 M^{-1} s^{-1} (data not shown).

An ATP chase experiment was also carried out with cross-linked acto-S1 (10 μM in S1 plus 50 μM [γ-^{32}P]ATP; results not illustrated). The progress curve was similar to that with myofibrils: there was a rapid rise (kinetics 31 ± 11 s^{-1}) followed by a steady state of 0.7 ± 0.2 s^{-1}.

Two additional features of the experiments with myofibrils deserve comment. First, the ATP chase method "works": there is a rapid transient of a large amplitude followed by a relatively slow k_{cat}. This suggests that, as with S1, $k_{-2} < k_{cat}$. But since the k_{cat} is much faster than with S1, the extrapolation of the steady-state rate to zero time is lower than the ATPase site concentration. We further discuss these points below. Second, the kinetics of the binding of ATP to myofibrils were monophasic; neither in the experiment illustrated in Figure 1b nor in several others, up to 100 μM ATP, could we discern a transient lag phase. This shows that on the time scale used ATP diffuses rapidly into myofibrils, in agreement with Sleep (1981).

A typical ATP chase experiment with myofibrils under single-turnover conditions is shown in Figure 2. There were two phases: a rapid phase (63% of the ATP hydrolyzed; k_{obs} = 9.9 s^{-1}) and a slow phase during which the remaining ATP was hydrolyzed. For technical reasons, the kinetics of the slow phase were not obtained but all the ATP was hydrolyzed in less than 2 s. It is noteworthy that, with cross-linked acto-S1 too, ATP was hydrolyzed in two stages in single-turnover experiments: about 50% rapidly and the remainder slowly (Biosca et al., 1985).

P_i Burst Experiments. Typical P_i burst experiments with S1 and myofibrils are illustrated in Figure 3. With both there were transient lag phases, presumably manifestations of the initial binding processes. With S1 this was followed by a P_i burst (kinetics, a function k and $k_3 + k_{-3}$) and finally a slow steady-state phase (not seen on the time scale used). With myofibrils too there was a P_i burst, but here the following

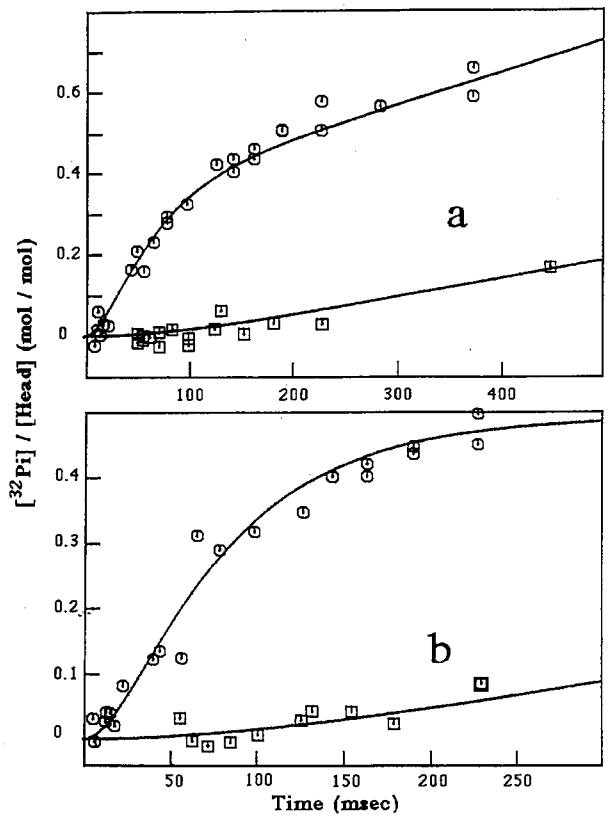

FIGURE 3: Time courses for P_i bursts with myofibrils (a) and S1 (b). The reaction mixtures were 10 μM myosin heads + 50 μM [γ-^{32}P]ATP (for myofibrils) and 3.3 μM S1 + 43 μM [γ-^{32}P]ATP (for S1). They were quenched in acid at the times shown, and the amount of [^{32}P]P_i was determined. For experimental conditions, see legend to Figure 1. For the points indicated with circles, the time scale is as indicated; for the points indicated with squares, (□) it is divided by ten.

steady state was rapid and the kinetic constant could only be obtained after computer simulations. The procedure used is described in the Discussion.

A P_i burst experiment was also carried out with cross-linked acto-S1 (10 μM in S1 and 50 μM [γ-^{32}P]ATP; curve not shown), and there was a large P_i burst and the kinetics were similar to those found with myofibrils (Figure 3b).

P_i Burst Experiments with Myofibrils in a Low Ionic Strength Buffer. As most kinetic work on acto-S1 has been carried out in buffers of low ionic strength, it was of interest to determined the behavior of activated myofibrils under such conditions.

A typical P_i burst experiment with myofibrils in a low ionic strength buffer is illustrated in Figure 4. There appears to be a small burst, but because of its low amplitude and the rapidity of the following steady state its kinetics were difficult to measure accurately. This result is very similar to that obtained with acto-S1 at a high actin concentration under the same conditions (Table II).

Discussion

From our experiments, reported both here and previously (Houadjeto et al., 1991), it appears that activated myofibrils are amenable to solution study using rapid reaction equipment. Our results concern the transients of the ATPase of myofibrils shortening under maximum velocity. Typically, the myofibrils contained 5–12 sarcomers, i.e., they were about 12–30 μm long, and yet mixing them rapidly with ATP in our equipment did not seem to cause any problem.

We obtained progress curves for myofibrillar ATPase by two methods (ATP chase and P_i burst) and at several ATP

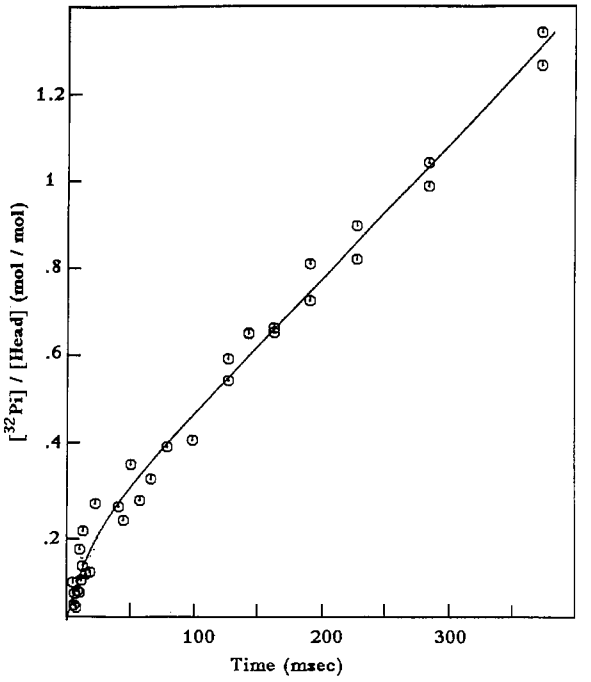

FIGURE 4: Time course for P_i burst with myofibrils in a low ionic strength buffer at 15 °C. The reaction mixtures (4.5 μM in myosin heads + 50 μM [γ-^{32}P]ATP) were quenched in acid and the amount of [^{32}P]P_i was determined. The buffer was 10 mM imidazole hydrochloride, pH 7, + 1.8 mM $MgCl_2$ + 0.1 mM $CaCl_2$.

Table II: Kinetic Constants for P_i Burst Experiments with Myofibrils and Acto-S1 at a Low Ionic Strength at 15 °C[a]

	P_i burst		steady-state rate (s^{-1})
	amplitude[b]	k (s^{-1})	
myofibrils	0.16 (±0.02)	53 (±18)	3.0 (±0.1)
acto-S1	0.15 (±0.03)	25 (±16)	4.5 (±0.6)

[a] The data for myofibrils are from Figure 5. The P_i burst experiment with acto-S1 was carried out at 40 μM actin (Tesi et al., 1990). [b] In units of moles of P_i per mole of myosin head.

concentrations (16, 32, 48, 75, and 100 μM). For technical reasons, we were unable to carry out experiments at higher ATP concentrations.

Do these results permit us to construct a reaction scheme for myofibrillar ATPase? It is noteworthy that whereas the steady-state rates of myofibrils and S1 were very different, their transient kinetics appear to be similar. Our procedure, therefore, was to fit the data obtained with myofibrils (using different methods of quenching and a large range of ATP concentrations) to the reaction scheme for S1 (Scheme I) by adjusting the different constants and ATPase site concentrations (true titration values). The constants thus obtained agreed well, and the consensus is given in Table I. We now discuss in more detail the validity and consequences of these constants.

Is the Binding of ATP to Myofibrils Tight? To us, this implies that $k_{-2} \ll k_{cat} \ll k_2$ (Scheme I). That $k_{-2} \ll k_2$ is clear from the ATP dependence: from it, $k_2 > 200$ s^{-1} and $k_{-2} < 2$ s^{-1}. A minimum value for k_{-2} of 0.04 s^{-1} (at 8 °C) is provided by the ATP and P_i exchange studies of Bowater and Sleep (1990) on muscle fibers and under ionic conditions similar to ours. Here we were unable to obtain a precise value for k_{-2}, but we can come to certain conclusions.

Chase experiments carried out under single-turnover conditions can give us an idea of the relative values of k_{-2}, k_2, and k_{cat}. In such experiments, all the [γ-^{32}P]ATP is turned over and if one assumes that only one site is involved with $k_{-2} <$

$k_{cat} < k_2$, the progress curves should be monophasic (a single exponential with all of the ATP hydrolyzed). A typical experiment is given in Figure 2. There was a rapid phase (63% of the ATP hydrolyzed) followed by a slow phase in which all of the remaining ATP was hydrolyzed. This was unexpected: is the slow phase a reflection of a k_{-2}? Probably not because computer simulation using Scheme I under the conditions of Figure 2 showed that the progress curve for M*·ATP + M**·ADP·P_i + P_i (i.e., the species determined in the chase; Barman & Travers, 1985) were monophasic: although the kinetics were somewhat sensitive to k_{-2}, slow phases were not obtained, even at large values for k_{-2} ($>k_4$). We discuss below a possible cause for the slow phase.

Now, consider the events that occur during the postchase period. Upon the injection of the excess unlabeled ATP, any free [γ-^{32}P]ATP (and the [γ-^{32}P]ATP in the collision complex M·ATP) is diluted out and lost for further reaction on the time scale of the chase (2 min). Since K_3 is large, (Table I) the concentration of radioactive M*·ATP is low and the predominant radioactive intermediate is M**·ADP·[^{32}P]P_i. The [^{32}P]P_i in this can escape by two routes: either via k_{-3} and k_{-2} (and the rapid equilibrium K_1) as [γ-^{32}P]ATP, which is diluted out by the unlabeled ATP, or via k_{cat} as [^{32}P]P_i. Only [^{32}P]P_i is determined by our assay method. Therefore, if $k_{-2} > k_{cat}$, not all the [γ-^{32}P]ATP would have been recovered in the 2-min postchase period. But in the single-turnover experiments, the [γ-^{32}P]ATP was recovered at >98%. This is good evidence that $k_{-2} < k_{cat}$.

That $k_{-2} < k_{cat}$ is further supported by the large amplitudes found in the ATP chase experiments under multiturnover conditions (e.g., Figure 1a). If $k_{-2} > k_{cat}$, the amplitudes would be consistently low or even nonexistent as during the ATP chase period the [γ-^{32}P]ATP in M*·ATP and the [^{32}P]P_i + ADP in M**·ADP·P_i would be released as free [γ-^{32}P]ATP as well as [^{32}P]P_i. We conclude that with rigor myofibrils $k_{-2} < k_{cat} \ll k_2$. This justifies our assumption in the computer simulations that by comparison with the other constants k_{-2} is near zero.

Can One Titrate Myofibrillar ATPase Sites by the ATP Chase Method? As argued in the Results section, with S1 the situation is clear: in the time range used for the ATP chase a plateau is reached which gives directly the concentration of the sites that bind tightly and hydrolyze ATP. Because of their high ATPase activities, this procedure could not be used with myofibrils. With these, the active site concentrations were obtained by adjustment in the fitting of the data. How valid is this procedure?

A noteworthy feature of the chase experiments is that there is a rather large variation in the titration amplitudes: from 0.6 to 0.9 mol of ATP bound/myosin head. With S1 too, there was a large variation in titration amplitudes (Tesi et al., 1989). Some of this variation is probably due to difficulties in obtaining accurate values for the myosin concentrations in myofibrillar preparations, but we estimate this error to be less than 10%. Further, the steady-state rates obtained were directly proportional to the amplitudes, again as for S1.

Thus, from the similarities with S1 we assume, as a working hypothesis, that one can titrate the ATPase sites in myofibrils by the ATP chase method.

What about the Fraction of Myofibrillar Heads That Are Not Titrated? From above, certain myofibrillar preparations contain rather large amounts of myosin heads that are not titrated by the ATP chase, i.e., that do not bind tightly and hydrolyze ATP.

This situation is obtained with myosin itself (Tesi et al., 1989). In that work we gave evidence that myosin and its proteolytic fragments contain two types of head whose relative amounts depend on the preparation: one hydrolyzes ATP by Scheme I, the other binds ATP loosely with no or very little hydrolysis. In particular, biphasic progress curves were obtained in ATP chase experiments under single-turnover conditions. The sizes of the amplitudes of the phases depended upon the preparation: the higher the ATPase site titration, the larger the amplitude of the fast phase.

With myofibrils, chase experiments under single-turnover conditions were also biphasic (Figure 2) which is apparently not explained by a k_{-2} (see above). Therefore, we suggest that, as with myosin, our preparations of myofibrils contain two types of sites for ATP. This would explain certain results of Sleep (1981) with myofibrils. He found that, under single-turnover conditions and at times well after all of the ATP should have been bound to the ATPase site, a certain amount of the ATP was released slowly into the medium. Sleep concluded that k_{-2} was similar to k_{cat}. However, his results could also be explained by the slow release of ATP from a weak site that does not hydrolyze ATP.

With S1—and its parent myosin—the cause of the variation in ATPase activity remains undetermined and the relationship site concentration versus ATPase activity represents results obtained over a period of several years. With myofibrils, too, the origin of the heterogeneity remains obscure. The similarity in behavior of materials as different as myofibrils and S1 is striking; this is particularly so when we take into account the very different methods used in their preparation. This heterogeneity is most probably explained by the presence of partially denatured myosin in the different preparations, but we cannot exclude the possibility that there are different ATP sites in the muscle itself [for a discussion of this problem, see Taylor et al. (1977)].

The Cleavage and Products Release Steps of Myofibrillar ATPase. Here we confined ourselves to an investigation of the chemical events that occur at the ATPase sites of S1, activated myofibrils, and acto-S1. The interactions between myosin and actin were not studied. We fitted our data according to Scheme I, and the constants obtained are summarized in Table I.

Under our experimental conditions there were large P_i bursts with S1 and especially with myofibrils (shown by a large K_3; Table I). This shows that with both the release of products step is rate limiting (k_4; Scheme I). Interestingly, under his experimental conditions (similar ionic strength but at 20 °C), Taylor (1990) obtained only a small P_i burst with myofibrils. At 15 °C, we too obtained a low P_i burst (Table II). We suggest that these differences in the sizes of the P_i bursts are due to a difference in the temperature sensitivity of the kinetics of two key steps of myofibrillar ATPase: the cleavage step ($k_3 + k_{-3}$, low sensitivity) and release of products step (k_4, high sensitivity). Thus, at low temperatures (e.g., 4 °C), $k_4 < k_3 + k_{-3}$, and at high temperatures (above 20 °C), $k_3 + k_{-3} < k_4$. Therefore, at the physiological 39.1 °C of the rabbit, the P_i burst would be expected to be small. We are at present exploring this possibility, but because of difficulties in separating the transient and two steady-state phases at temperatures above 4 °C (Houadjeto et al., 1991) this is not easy.

At 4 °C (Table I), there is only one major difference between the kinetics of the organized myofibrils and the dispersed molecules: the release of products is much faster with the myofibrils. Thus, in practice the influence of actin upon the steps preceding the release of products is low. This is in accord

with the early conclusions of Lymn and Taylor (1971), based on studies with actomyosin. They proposed that, following the binding of ATP, the effect of actin is low ("dissociation") but that after the cleavage step it becomes high ("reassociation").

But in addition to the large effect of actin upon the release of products steps, there could be a subtle change at the cleavage step (Table I): it appears, therefore, that this step in myosin ATPase is perturbed somewhat in myofibrils. We are studying further this possibility.

It is noteworthy that the kinetics (transient and steady state) of myofibrils and cross-linked acto-S1 are so similar. Of course, unlike with acto-S1, with cross-linked acto-S1 full ATP induced dissociation does not occur. Nevertheless, upon the addition of ATP to cross-linked acto-S1 there is a change in the structure of the myosin heads (Craig et al., 1985), and this might be enough to accelerate the release of products steps.

It was not possible to compare the kinetics of myofibrils and acto-S1 in our relatively high ionic strength buffer. In a low ionic strength buffer this was feasible, and from Table II the kinetics of the two systems are seen to be similar.

Possible Effect of Mechanical Constraint upon Myofibrillar ATPases. In the systems studied here (myofibrils, acto-S1, and cross-linked acto-S1), there is little restraint during the ATPase cycle; i.e., little work is carried out during ATP hydrolysis. In particular, since the myofibrils are not anchored, they contract at maximum velocity near zero load. Would their kinetics have been different had they been held?

There is evidence that their steady-state rates would have been slower. Thus, Glyn and Sleep (1985) obtained a steady-state rate of 0.85 s^{-1} at 15 °C with myofibrils that had been cross-linked chemically. These modified myofibrils did not contract upon the addition of ATP. Their low ATPase activity is consistent with the 1.9 s^{-1} (at 19 °C) obtained by Ferenczi et al. (1984) with muscle fibers held isometrically. These rates are low compared with our 1.7 s^{-1} at 4 °C for unheld myofibrils (Table I). Further, from their ^{18}O work, Bowater et al. (1989) concluded that with muscle fibers k_{-2} is close to k_{cat}. Thus, it appears that with the held fibers k_{-2} is considerably larger than with the unheld myofibrils.

These differences are in accord with Huxley (1980, 1988), namely, that the rates of certain chemical reactions in muscle fibers are governed by the mechanical conditions.

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