Early Steps of the Mg^{2+}-ATPase of Relaxed Myofibrils. A Comparison with Ca^{2+}-Activated Myofibrils and Myosin Subfragment 1†

C. Herrmann, M. Houadjeto, F. Travers, and T. Barman*

INSERM U128, CNRS, BP 5051, 34033 Montpellier Cedex, France

Received January 21, 1992; Revised Manuscript Received May 20, 1992

ABSTRACT: The early steps of the Mg^{2+}-ATPase activity of relaxed rabbit psoas myofibrils 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 product steps by the P_i burst method. The data obtained were interpreted by

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

where M represents the myosin heads with or without actin interaction. This work is a continuation of our study on Ca^{2+}-activated myofibrils [Houadjeto, M., Travers, F., & Barman, T. (1992) *Biochemistry 31,* 1564–1569]. Here the constants obtained with relaxed myofibrils were compared with those with activated myofibrils and myosin subfragment 1 (S1). We find that whereas Ca^{2+} increases 80× the release of products (k_4), it has little effect upon the kinetics of the initial binding and cleavage steps. As with activated myofibrils and S1, the second-order binding constant for ATP (k_2/K_1) was about 1 μM^{-1} s^{-1} and the ATP was bound very tightly. With activated myofibrils, it was difficult to obtain an estimate for the k_{off} for ATP (k_{-2}) but it is $\ll k_{cat}$. Here with relaxed myofibrils we estimate $k_{-2} < 8 \times 10^{-4}$ s^{-1}, which is considerably smaller than k_{cat} (0.019 s^{-1}) and also previous estimates for this constant. The overall K_d for ATP to relaxed myofibrils is less than 8×10^{-10} M. With S1 this K_d is about 10^{-11} M. The kinetics of the cleavage step with relaxed and activated myofibrils are probably similar but different from those with S1. From our previous work it appears that K_3 is larger with activated myofibrils than with S1 but it was difficult to obtain an estimate for this constant. Here with relaxed myofibrils K_3 was estimated at 6, which is considerably larger than the 1.7 found with S1.

Muscle contraction depends on the cyclic interaction of actin and myosin, the energy for which is supplied by the hydrolysis of ATP[1] by the myosin heads. It is thought that the contractile process is modulated by the various intermediates on the ATPase reaction pathway as illustrated by the classical cross-bridge model [e.g., Huxley (1988)]. Therefore, for a full understanding of muscle contraction, this pathway must be elucidated. Ideally one should study directly the ATPase of muscle fibers, but this is very difficult. An approach is to use caged compounds [e.g., Hibberd and Trentham (1986) and Homsher and Millar (1990)], a novel technique that shows great promise.

Most muscle kineticists have directed their attention toward the kinetics of isolated myosin and reconstituted actomyosin ATPase [e.g., Trentham et al. (1976), Taylor (1979), Eisenberg and Hill (1985) and Geeves (1991)]. For technical reasons the bulk of these studies was carried out at nonphysiologically low ionic strengths. Also, it may be hazardous to extrapolate from studies carried out on dispersed molecules to the organized muscle fibers.

A compromise is to experiment with myofibrils. These are the functional contractile units of muscle, and yet they are small enough for study in rapid reaction machines. Being organized, they can be studied at physiological ionic strengths. However, they are not anchored and when Ca^{2+} activated at excess ATP they overcontract, leading to an apparent loss of structure. Studies have been carried out on the overall myofibrillar ATPases. Taylor (1990) has carried out P_i burst experiments with Ca^{2+}-activated myofibrils. We have already discussed these works (Houadjeto et al., 1992).

Recently we began a detailed study on the ATPases of myofibrils, in particular on the kinetics of the early steps of the reaction pathways. The study was carried out in a buffer of near-physiological ionic strength but at 4 °C as at higher temperatures the kinetics were very rapid. We interpreted our data by a shortened version of the Bagshaw–Trentham scheme (Trentham et al., 1976), where M represents the myosin heads (Scheme I).

Scheme I

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

The ATPase of myofibrils is studied either in the absence or in the presence of Ca^{2+}. They are mixed with [γ-^{32}P]ATP in a rapid flow quench apparatus, and the production of [^{32}P]-P_i is studied on the millisecond to several seconds time scale. This, of course, is not the situation in the muscle where the contractile process is initiated by Ca^{2+} rather than ATP [for a discussion of this problem, see Ferenczi (1986)]. Now, in the absence of ATP, the myosin heads in the myofibrils are attached to the thin filament whether Ca^{2+} is present or not;

† This work was supported by a grant from the EEC (stimulating Action). C.H. and M.H. were supported by INSERM fellowships.

[1] Abbreviations: ATP, adenosine 5′-triphosphate; P_i, inorganic orthophosphate; S1, myosin subfragment 1; Tris, tris(hydroxymethyl)-aminomethane; DTT, dithiothreitol; PMSF, phenylmethylsulfonyl fluoride; EDTA, (ethylenedinitrilo)tetraacetic acid; EGTA, ethylene glycol bis(2-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid.

0006-2960/92/0431-8036$03.00/0

i.e., they are in "rigor" [Payne and Rudnick (1989) and references cited therein]. It is only upon the addition of ATP that the heads become rapidly detached and relaxation occurs. In the presence of Ca^{2+} this detachment lasts a short time only, but in its absence it is long lasting. Here, for convenience sake, we term myofibrils in the absence of Ca^{2+} "relaxed". Upon the addition of Mg^{2+}-ATP these do not contract, and the ATP is hydrolyzed very slowly. In the presence of Ca^{2+} and ATP the myofibrils contract and hydrolyze rapidly ATP. These we term "activated" myofibrils.

In our study we addressed ourselves to four main questions. First, what is the ATPase activity of contracting myofibrils? Second, how tightly do they bind ATP? Third, does the cleavage step proceed while the heads are attached to the thin filament? Finally, how does Ca^{2+} affect the transient kinetics of myofibrillar ATPase?

We have already obtained answers to certain of these questions. Thus, in Houadjeto et al. (1991), we found that a k_{cat} can be obtained during the first second after mixing activated myofibrils with an excess of ATP. In Houadjeto et al. (1992) we provided a transient kinetic study on activated myofibrils. We showed that they bind ATP tightly, but because of their large k_{cat} we could not estimate the k_{off} for ATP (k_{-2}, Scheme I). Interestingly, there appeared to be a subtle difference in the kinetics of the cleavage steps of activated myofibrils and myosin, but because of the rapid k_{cat} with the myofibrils this difference was uncertain. Thus, with activated myofibrils we could not come to a clear-cut answer to our third question.

Here we addressed ourselves to the transient kinetics of relaxed myofibrils, a study which enabled us to approach our fourth question. But it also allowed us to obtain an upper estimate for k_{-2}, which we show is considerably smaller than k_{cat}. Thus, as myosin, relaxed myofibrils bind ATP very tightly. Further, our study allowed us to obtain with more precision the kinetics of the cleavage step with myofibrils, as in the absence of Ca^{2+} k_{cat} is low. We show that the kinetics of this step with relaxed and activated myofibrils are probably similar but different from those with myosin.

MATERIALS AND METHODS

Proteins and Reagents. Myofibrils were prepared from rabbit psoas muscle following Knight and Trinick (1982) except that the washing buffer was 50 mM KCl, 50 mM Tris pH 7 (HCl), 5 mM EDTA, 5 mM EGTA, 1 mM DTT, and 1 mM PMSF. Myosin subfragment 1 (S1) was prepared as in Biosca et al. (1984). [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 by 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 EGTA, 5 mM magnesium acetate, and 50 mM Tris adjusted to pH 7.4 with acetic acid.

Rapid Flow Quench Experiments. Two types of experiment were carried out. In *ATP chase experiments*, S1 or myofibrils plus [γ-^{32}P]ATP reaction mixture are first quenched in a large excess of unlabeled Mg^{2+}-ATP (50 mM) in a rapid flow quench apparatus, incubated on ice for 2 min, and then quenched in 22% trichloroacetic acid, and the [^{32}P]P_i is determined. In these experiments, one measures the kinetics of the binding of ATP to the myosin heads with $k = k_2[ATP]/K_1 + [ATP]$. Their success depends upon the binding being essentially irreversible; i.e., that $k_{-2} \ll k_{cat} \ll k_2$ (Scheme I). For a full discussion of the ATP chase technique, see Barman and Travers (1985). In *P_i burst experiments*, reaction mixtures are quenched directly in acid, and the [^{32}P]P_i is determined. Here one obtains information on the kinetics of the cleavage and release of products steps (k_3, k_{-3}, and k_4, Scheme I).

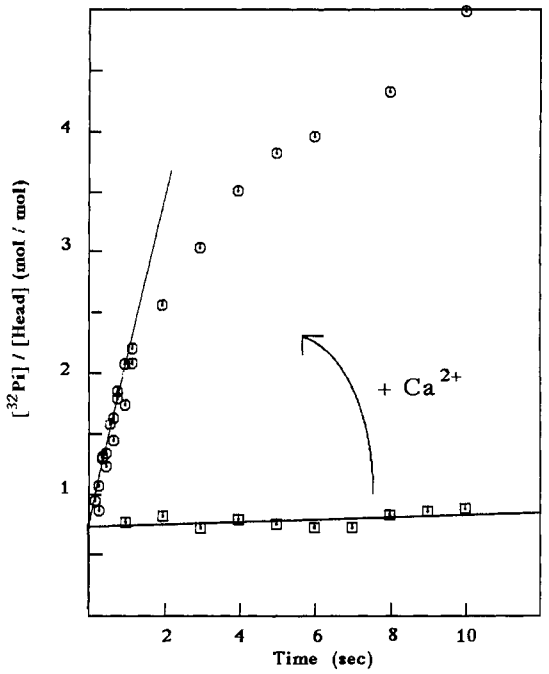

FIGURE 1: Steady-state time courses for relaxed myofibrils (□) and the same myofibrils activated by Ca^{2+} (⊙) at 4 °C. The reaction mixtures (3 μM in myosin heads + 32 μM [γ-^{32}P]ATP) were quenched in acid, and the [^{32}P]P_i was determined. The buffer was 50 mM Tris–acetate, pH 7.4, 0.1 M potassium acetate, 5 mM KCl, 5 mM magnesium acetate, and 2 mM EGTA. To activate, 3 mM $CaCl_2$ was added.

Both types of experiments were carried out in thermostatically controlled rapid flow quench apparatuses constructed in this laboratory (Barman & Travers, 1985). The data were treated following Houadjeto et al. (1992).

RESULTS

The Myofibrils Are Well Regulated. As illustrated in Figure 1, the ATPase activity of relaxed myofibrils was low (0.015 s^{-1}), but when an excess of Ca^{2+} was added to a portion of the same myofibrillar preparation, there was a dramatic increase in activity to 1 s^{-1}. The initial P_i burst amplitudes were large and they appear to be independent of the presence of Ca^{2+}. Large P_i bursts with relaxed myofibrils were also obtained by White (1985) and Miyato et al. 1989). The progress curve for the activated myofibrils was very similar to that with myofibrils that had not been treated with EGTA (Houadjeto et al., 1991). These results show that the myofibrils were well regulated and that the effect of EGTA is reversible. From observations under the microscope, relaxed myofibrils did not contract upon the addition of ATP but when Ca^{2+} was also added they did.

Tight Binding of ATP to Relaxed Myofibrils. A typical ATP chase experiment with relaxed myofibrils at 45 μM ATP is illustrated in Figure 2a. There was a rapid rise of bound ATP of amplitude 0.58 mol of ATP/mol of myosin head and kinetics $k = 39$ (±5) s^{-1}, which was followed by a steady-state

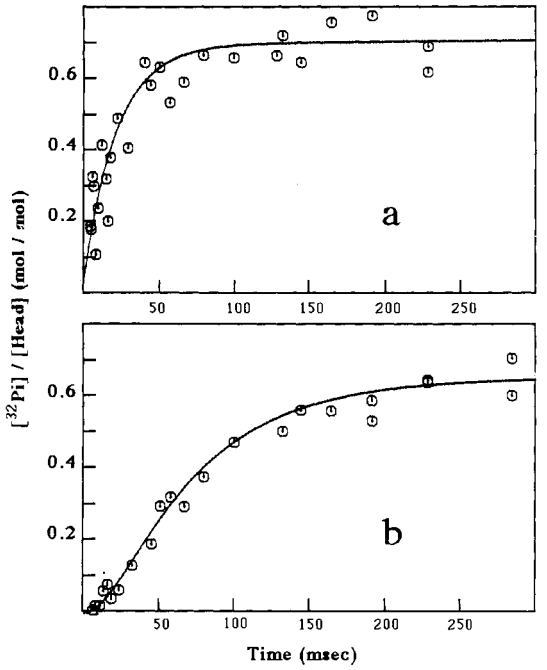

FIGURE 2: Time courses for the binding of ATP by the ATP chase (a) and for a P_i burst experiment (b) with relaxed myofibrils. The reaction mixtures (4.8 μM in myosin heads + 45 μM [γ-^{32}P]ATP) were quenched in cold ATP (a) or in acid (b), and the [^{32}P]P_i was determined. The curves were obtained by computer fitting using Scheme I and the constants in Table I.

Table I: Kinetic Constants of the Early Steps of Myofibrillar and S1 ATPases at 4 °C As Interpreted by Scheme I[a]

constant	myofibrils		S1
	+Ca^{2+} [b]	−Ca^{2+}	
k_2/K_1 (μM^{-1} s^{-1})	1 (±0.2)	1 (±0.2)	1 (±0.2)
$k_3 + k_{-3}$ (s^{-1})	18 (±2)	18 (±2)	16 (±3)
K_3	"large"	6 (±0.7)	1.7 (±0.2)
k_4 (s^{-1})[c]	1.7	0.021	0.027
k_{cat} (s^{-1})	1.7 (±0.2)	0.019 (±0.002)	0.017 (±0.002)

[a] The buffer was 50 mM Tris, 0.1 M potassium acetate, 5 mM KCl, and 2 mM magnesium acetate (rigor myofibrils or S1) or 5 mM magnesium acetate (relaxed myofibrils), pH 7.4. Under rigor conditions $CaCl_2$ was 0.1 mM, and under relaxing conditions EGTA was 2 mM. [b] Houadjeto et al. (1992). [c] $k_4 = k_{cat}(1 + K_3)/K_3$.

rate of 0.011 s^{-1} (not shown in figure). This experiment suggests that, as with S1 and activated myofibrils (Houadjeto et al., 1992), the ATP chase "works" with relaxed myofibrils; i.e., that $k_{-2} < k_{cat} < k_2$. If we assume that the amplitude is equal to the ATPase site concentration, then $k_{cat} = 0.019$ s^{-1} (i.e., steady-state rate/chase amplitude).

A further chase experiment carried out at 31 μM ATP gave $k = 32$ (±3) s^{-1}. Taken together, these experiments show that the second-order binding constant (k_2/K_1) for ATP to relaxed myofibrils is about 1 μM^{-1} s^{-1}, which is very similar to those found with activated myofibrils and S1 (Table I).

That k_{-2} is small was confirmed by carrying out an ATP chase experiment under single-turnover conditions (i.e., [ATP] ≪ [ATPase sites]). This is illustrated in Figure 3. The key feature of the experiment concerns the fate of the [γ-^{32}P]-ATP in M*·ATP and [^{32}P]P_i in M**·ADP·P_i: during the chase period, both can escape as [γ-^{32}P]ATP (via k_2 and K_1) and [^{32}P]P_i (via K_3 and k_4) as indicated in Scheme II, where O indicates ^{32}P-labeled species.

On the time scale of the chase (2 min), the escaped [γ-^{32}P]-ATP is lost to further reaction and only the [^{32}P]P_i is

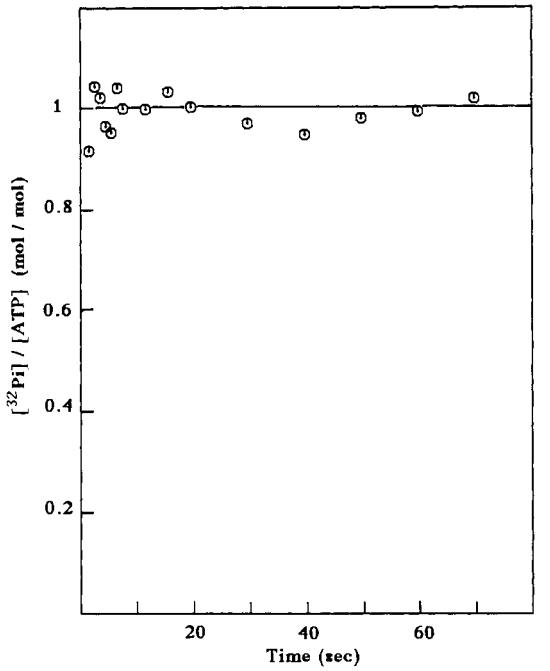

FIGURE 3: ATP chase experiment with relaxed myofibrils under single-turnover conditions. The reaction mixtures (7.5 μM in myosin heads + 0.1 μM [γ-^{32}P]ATP) were quenched in cold ATP, incubated on ice for 2 min and quenched in acid, and the [^{32}P]P_i was determined.

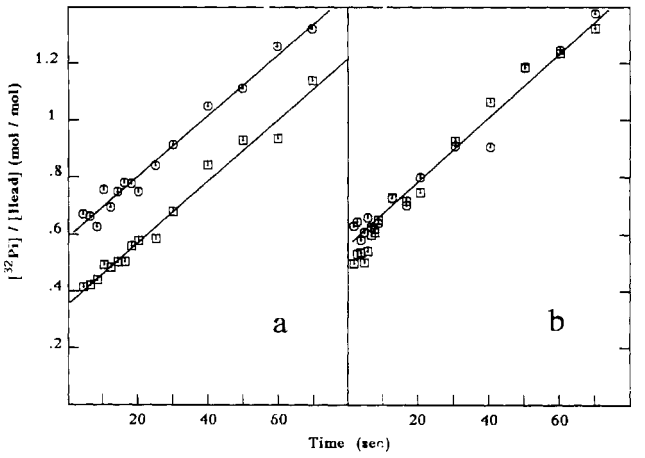

FIGURE 4: ATP chase and P_i burst experiments with S1 (a) and relaxed myofibrils (b) in the steady state. The reaction mixtures (3 μM in myosin heads + 10 μM [γ-^{32}P]ATP) were quenched in cold ATP (⊙) or acid (⊡), and the [^{32}P]P_i was determined.

Scheme II

$$M + ATP^{O} \underset{}{\overset{K_1}{\rightleftharpoons}} M{\cdot}ATP^{O} \overset{k_{-2}}{\dashrightarrow} M^{*}{\cdot}ATP^{O} \overset{K_3}{\rightleftharpoons}$$

$$M^{**}{\cdot}ADP{\cdot}P_i^{O} \xrightarrow{k_4} M + ADP + P_i^{O}$$

determined by our method. This determined [^{32}P]P_i is given by

$$[^{32}P]P_i/\text{total } [\gamma\text{-}^{32}P]ATP = k_{cat}/(k_{cat} + k_{-2})$$

In the experiment, more than 96% of the [γ-^{32}P]ATP was recovered as [^{32}P]P_i after a reaction time of less than 2 s, i.e., after a time much less than $1/k_{cat}$ (about 40 s). Since k_{cat} = 0.019 s^{-1}, we estimated k_{-2} to be smaller than 8×10^{-4} s^{-1}.

From our estimates of k_2/K_1 and k_{-2}, the overall dissociation constant for ATP (K_1K_2) to relaxed myofibrils is less than 8×10^{-10} M. With S1, this constant is about 10^{-11} M (Goody et al., 1977).

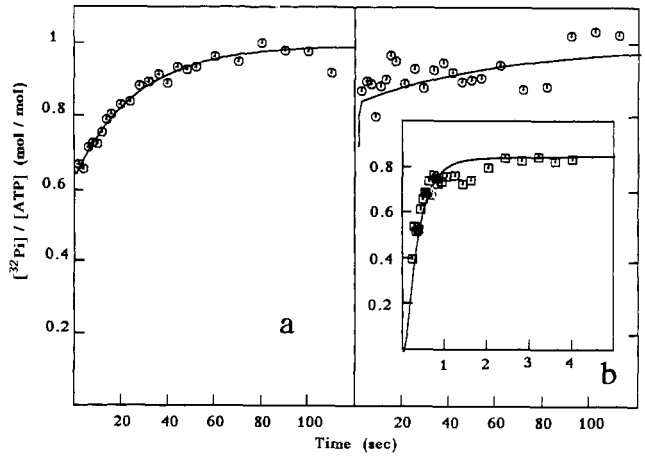

FIGURE 5: P_i burst experiments with S1 (a) and relaxed myofibrils (b) under single-turnover conditions. The inset shows the kinetics of the initial rise. The reactions mixtures (7.5 μM in myosin heads + 0.1 μM [γ-^{32}P]ATP) were quenched in acid, and the [^{32}P]P_i was determined.

We conclude that as for S1 (Trentham et al., 1976) and acto-S1 (Biosca et al., 1985) $k_{-2} \ll k_{cat} \ll k_2$, i.e., that by the ATP chase method one can titrate the ATPase sites in relaxed myofibrils.

ATP Chase and P_i Burst Experiments in the Steady State. ATP chase and P_i burst progress curves for relaxed myofibrils and S1 are illustrated in Figure 4. These experiments allowed for estimates for the equilibrium constant for the cleavage steps (K_3 = [M**·ADP·P_i]/[M*·ATP]). Thus, the amplitudes in the chase experiments give the ATPase site concentrations (i.e., M*·ATP + M**·ADP·P_i) and those in the P_i burst, M**·ADP·P_i. We estimate K_3 = 1.6 for S1 but much higher for relaxed myofibrils.

From the ATP chase experiments for S1, k_{cat} = 0.017 s^{-1}, and for relaxed myofibrils it is 0.019 s^{-1}. It appears, therefore, that whereas the k_{cat} values for S1 and relaxed myofibrils are similar, the equilibrium constant for the cleavage steps are different.

P_i Burst Experiments under Single-Turnover Conditions. When K_3 is very different from 1, it is difficult to obtain with precision from multiturnover experiments. A better way is to carry out P_i burst experiments under single-turnover conditions in the $1/k_{cat}$ time range (Bagshaw & Trentham, 1973). Such experiments are precise since all of the [γ-^{32}P]-ATP is turned over and provided that [ATPase sites] $\gg$ [ATP] they can yield estimates for K_3 and k_{off} for the products that are independent of the ATPase site concentration. However, the success of single-turnover experiments depends upon there being no interference from secondary ATP sites. This interference, which is sensitive to the experimental conditions, can be tested for carrying out ATP chase experiments under single-turnover conditions on the $1/k_{cat}$ time scale. Thus, under certain conditions with S1 there were biphasic ATP chase curves (Tesi et al., 1989).

Under the conditions used here, activated myofibrils too gave a biphasic chase curve (Houadjeto et al., 1992). That experiment was carried out on the millisecond time scale, which with the rapid ATPase activity of the activated myofibrils is in the $1/k_{cat}$ time range. As shown above, in single-turnover chase experiments with the slower relaxed myofibrils, all of the ATP was bound in less than 2 s. Thus, at times greater than 2 s any secondary ATP site should not interfere in P_i burst experiments.

A typical P_i burst experiment with S1 under single-turnover conditions is illustrated in Figure 5a. There was a rapid initial burst phase of amplitude, 0.63 mol of P_i/mol of ATP, equal to $K_3/1 + K_3$. Thus, K_3 = 1.7, which agrees well with the 1.6 obtained under multiturnover conditions. The rapid burst was followed by a slow exponential of 0.04 s^{-1} leading to complete hydrolysis of the ATP.

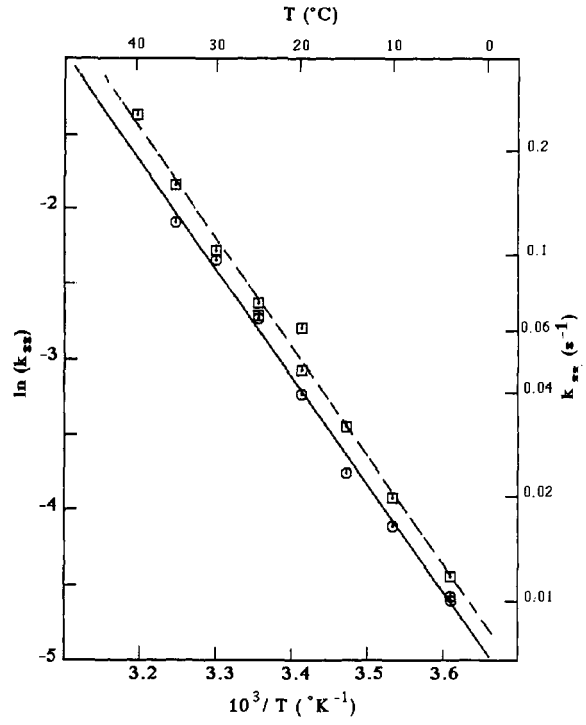

FIGURE 6: Temperature dependencies of the steady-state ATPases of relaxed myofibrils (⊙) and S1 (⊡).

With relaxed myofibrils, the initial burst phase was large and it was difficult to obtain the kinetics of the slow rise leading to the complete hydrolysis of the ATP (Figure 5b). The amplitude of the rapid rise was 0.85 mol of P_i/mol of ATP from which K_3 = 6, which is considerably larger than the 1.7 found with S1. The kinetics of the slow rise appears to be similar to that with S1 (Figure 5a). The inset in Figure 5b shows the kinetics of the initial rapid rise with relaxed myofibrils (3.2 ± 0.1 s^{-1}).

Similarity in Transient Kinetics of the Cleavage Steps with S1 and Relaxed Myofibrils. A P_i burst experiment was carried out with the same myofibrillar preparation and under the same conditions as those used in the chase experiment, and the result is given in Figure 2b. The transient lag phase is presumably a manifestation of the initial binding of ATP. Both the kinetics and the amplitude of the burst were similar to those found with activated myofibrils (Houadjeto et al., 1992) except, of course, that the following steady state was much slower. Accordingly, the data points in Figure 2b were fitted using Scheme I and a large K_3 (6) and the value of k_3 + k_{-3} found for rigor myofibrils (see Table I).

Similarity in Temperature Dependencies of the k_{cat} of S1 and Relaxed Myofibrils. As shown in Figure 6, the temperature dependencies of the Mg^{2+}-ATPases of S1 and relaxed myofibrils are similar. In the temperature range used (4–35 °C), the variation of ln k_{cat} as a function of the inverse of absolute temperature (K) was linear, with the $\Delta H^{\ddagger}$ for S1 58.0 (±2) kJ/mol and for relaxed myofibrils 57.3 (±1.7) kJ/mol. The similarity of the two energies of activation suggests that S1 and relaxed myofibrillar ATPases have similar rate-limiting constants for ATP hydrolysis.

From the dependence with myofibrils, the k_{cat} at the body temperature of the rabbit (39.1 °C) is 0.34 s^{-1}. At 25 °C it

is 0.14 s^{-1}, which is in reasonable agreement with the 0.2 s^{-1} found by Goodno et al. (1978).

DISCUSSION

An important question in muscle contraction concerns the mode of interaction of the cross bridges (myosin heads) with the thin filament (regulated actin) and how this interaction affects the ATPase reaction pathways of the heads. In particular, there are questions concerning the role of Ca^{2+}. Ca^{2+} increases dramatically the overall ATPase of muscle fibers (as well as that of myofibrils and regulated acto-S1), but its precise effect upon the myosin head–thin filament interaction remains obscure. It appears that, in the absence of ATP, the heads are attached, whether Ca^{2+} is present or not [Brenner et al. (1982), Cooke (1986) and Payne and Rudnick (1989); but see Miyata et al. (1989)]. When ATP is added, the heads detach rapidly but Ca^{2+} has little effect on the kinetics of this. Goldman (1987) suggested that the only steps that are accelerated significantly by Ca^{2+} are those involved in the release of products. Here we confirm this suggestion by showing that Ca^{2+} does not seem to affect significantly the initial steps of myofibrillar Mg^{2+}-ATPase. The relevant kinetic constants are summarized in Table I. Therefore, if we use as a criterion the comparison of the kinetics of the binding and the cleavage steps, relaxed and activated myofibrils appear to be identical. We discusse below in more detail the myofibrillar Mg^{2+}-ATPases and we compare these with that of S1 ATPase.

Binding of ATP. From the results of the ATP chase experiments with the myofibrils [relaxed here, activated in Houadjeto et al. (1992)] and S1 we conclude the following. First, for all three materials the binding processes appear to be essentially irreversible; i.e., $k_2 \ll k_{cat} \ll k_2$. Thus, by the ATP chase method one can titrate the ATPase sites in myofibrils as well as in S1. Second, the second-order binding constants (k_2/K_1) were identical at about 1 μM^{-1} s^{-1}. Of course, it does not follow that the individual constants K_1 and k_2, with the three materials are identical; to obtain these, saturation kinetics must be obtained, which was not possible under our experimental conditions. For instance, in a buffer containing 40% ethylene glycol, with S1 and acto-S1 the ratios k_2/K_1 were identical but the individual constants were different (Biosca et al., 1984). Finally, transient lag phases were not discerned in any of the chase experiments, even at high ATP concentrations (i.e., where the binding kinetics are fast). This confirms that ATP diffuses rapidly to the ATPase sites in myofibrils (Sleep, 1981).

The low value for k_{-2} with relaxed myofibrils is noteworthy: $<8 \times 10^{-4}$ s^{-1} compared with a k_{cat} of 0.019 s^{-1}. Thus, under our experimental conditions $k_{cat}/k_{-2} > 24$.

Our estimate for k_{-2} with relaxed myofibrils is considerably lower than previous ones with other muscle systems. Thus, Rosenfeld and Taylor (1984) carried out ATP chase experiments with relaxed regulated acto-S1 under single-turnover conditions and concluded that k_{-2} and k_{cat} are similar: 0.8 and 0.6 s^{-1}, respectively. Bowater et al. (1989) carried out oxygen-exchange studies with relaxed skinned fibers and estimated k_{-2} = 0.63 s^{-1} and k_{cat} = 0.04 s^{-1}.

What is the cause for these differences? They are large and unlikely to be explained by the different experimental conditions used [Rosenfeld and Taylor (1984), 20 °C, very low ionic strength; Bowater et al. (1989), 24 °C, ionic strength 20 mM]. We note that Bowater et al. (1989) carried out their experiments at a much higher ATP concentration (8 mM) than we did (micromolar range). A more likely cause is that there is a complication arising from the presence of secondary ATP sites in the different muscle systems. It cannot be excluded that these sites have low ATPase activities. The sites were detected in ATP chase experiments under single-turnover conditions with S1, acto-S1, and activated myofibrils [for a discussion of this problem, see Tesi et al. (1989) and Houadjeto et al. (1992)]. Shukla et al. (1983) and Evans (1988) have already pointed out the danger of secondary ATPase sites in the interpretation of certain types of studies.

Cleavage Step. In the P_i burst experiments, the amplitudes were considerably larger with the myofibrils than with S1 (Figures 4 and 5). Before we can attribute this difference to different values for K_3—the equilibrium constant for the cleavage step—we must consider another explanation.

Our P_i burst experiments were carried out under two conditions—single turnover and multiturnover. Consider first the single-turnover experiments at very low ATP concentrations (Figure 5). Bremel and Weber (1972) found that at low ATP and in the absence of Ca^{2+}, the Mg^{2+}-ATPase of myosin was activated by regulated actin. Moss and Haworth (1984) showed that skinned rabbit skeletal muscle fibers contract at low levels of Mg^{2+}-ATP in the absence of Ca^{2+}. In these experiments only a few myosin heads interact with ATP, the majority being still attached to the thin filament. Bremel and Weber (1972) proposed that these attached heads activate the entire thin filament, which leads to the rapid cycling of the few heads that contain bound ATP and to an acceleration of the release of products from these.

It is possible, therefore, that the large amplitude in the single-turnover experiment with relaxed myofibrils has two components: a P_i burst due to $M^{**}\cdot ADP\cdot P_i$ followed by a rapid liberation of free P_i due to a transient activation of the cross bridges. There are two reasons why we think that this is unlikely. First, consider the rate constant for the initial rise in the single-turnover experiment (inset in Figure 5b). In a situation where there is no transient activation, this overall rate constant (k_{obs}) should be a manifestation of the binding of ATP (K_1 and k_2, Scheme I) and the cleavage step (k_3 and k_{-3}). With a second-order binding constant of 1 μM^{-1} s^{-1} and an ATPase site concentration of 5.6 μM, the kinetics of the binding process is 5.6 s^{-1} (in the experiment the myosin heads, 7.5 μM, titrated 75% in active sites). Since the kinetics of the cleavage step is 18 (±2) s^{-1} (Table I), the overall k_{obs} = 4.2 (±0.3) s^{-1}. This is in reasonable agreement with the 3.2 (±.1) s^{-1} obtained.

Second, large amplitudes were also obtained in the multiturnover experiments (e.g., Figure 4). In these, the concentrations of ATP were larger than those of the myosin heads and any ATP-induced activation was unlikely. Further, here the kinetics of the burst phases could be fitted to a simple P_i burst due to the formation of $M^{**}\cdot ADP\cdot P_i$ (Figure 2b). We conclude that the large P_i bursts with relaxed myofibrils are due to a large K_3.

In our experiments, then, we could not detect any transient activation of the cross bridges. This is as expected—the conditions were single turnover and with a large K_3 any activation would be difficult to perceive. In their experiments, Bremel and Weber (1972) included an ATP-regenerating system and ATPase activities in the steady state were measured.

With activated myofibrils too, K_3 appears to be large but because of the rapid k_{cat} it was difficult to obtain an estimate for it (Houadjeto et al., 1992). It is noteworthy that whereas the kinetics ($k_3 + k_{-3}$) of the P_i burst with myofibrils (relaxed or activated) and S1 are similar, the equilibrium constants K_3

(k_3/k_{-3}) are different. This is at variance with Miyata et al. (1989), who concluded that the overall kinetics of the cleavage steps of relaxed myofibrils and S1 are identical. Rosenfeld and Taylor (1984) concluded that K_3 is smaller with acto-S1 than with S1 (0.45 and 1.5, respectively), but their experiments were carried out in a very weak ionic strength buffer and at 20 °C.

Is the modification of the cleavage step of myosin ATPase when in myofibrils due to an interaction with actin? This would be in accord with Sleep and Hutton (1981), who proposed that at high actin concentrations the cleavage step in acto-S1 takes place without dissociation. Unfortunately, under most experimental conditions it is difficult to obtain the kinetics of the cleavage step of acto-S1 at high actin and therefore to compare them with those with S1 ATPase [for a discussion, see Tesi et al. (1990)]. However, under cryoenzymic conditions (−15 °C with 40% ethylene glycol as antifreeze), the interaction of S1 with actin is strong, even in the presence of ATP (Tesi et al., 1991). In that work we concluded that at high actin, K_3 is larger with acto-S1 than with S1 and yet the acto-S1 appeared to be wholly dissociated for the duration of the cleavage step. To explain this, we suggested that following the ATP-induced dissociation the S1 retains its actin-induced structure for sufficient time for the cleavage step to be modified. Thus, we cannot exclude the possibility that in myofibrils the cleavage step changes not because the heads are attached but because they have retained their actin-induced structures.

Release of Products Steps and k_{cat}. At 4 °C, with both S1 and myofibrils, it is the release of products steps that determines the overall k_{cat}. With myofibrils there are two problems in obtaining accurate k_{cat} values. First, the necessary myosin head and, therefore, ATPase site concentrations are not easily available. Here we titrated the ATPase sites directly by the cold ATP chase method. Second, because of damage to the regulatory proteins, the relaxation of the myofibrils may not always be complete. We cannot exclude this possibility: our k_{cat} values may be overestimated. However, they were reproducible and further, very similar to those found with S1. This is exemplified by the Arrhenius plots of the two activities (Figure 6). It seems unlikely that this similarity is a coincidence.

With myosin it is known that the release of products from M**·ADP·P_i takes place in two steps with the P_i coming off before the ADP. The rate of release of P_i can be determined specifically by carrying out a P_i burst experiment under single-turnover conditions (Trentham et al., 1976). Thus, in Figure 5a the exponential following the rapid rise in P_i is given by $k_aK_3/1 + K_3$, where k_a is the rate constant for the release of P_i. Here the exponential is given by $k_{obs} = 0.04\ s^{-1}$ and $K_3 = 1.7$, from which $k_a = 0.064\ s^{-1}$.

Because of the large values of K_3 with myofibrils, we were unable to study specifically the release of P_i. Instead, we interpreted our results by a simple scheme in which the two products are released in one step (Scheme I). As with S1, it is the release of product step that is rate limiting with myofibrillar ATPase: in both cases there were large P_i bursts. Thus, the overall k_{cat} is largely a reflection of k_4. This constant ($0.021\ s^{-1}$) is considerably smaller than k_{-3} ($1.1\ s^{-1}$, calculated from Table I), the rate constant for the reversal of the cleavage step. Hibberd et al. (1985) carried out oxygen-exchange studies with relaxed muscle fibers, and they too concluded that the rate constant for the reversal of the cleavage step is greater than that for the P_i release.

It is remarkable that k_{cat} is so similar for relaxed myofibrils and S1 and, further, that their energies of activation are virtually identical (Figure 6). Does this mean that the kinetics of the steps abridged by k_4 in Scheme I (i.e., the sequential release of P_i and ADP) for S1 and relaxed myofibrils are identical? Probably not. There is evidence that the release of ADP may proceed with different kinetics in the two materials. Thus, Biosca et al. (1988) showed that ADP binds much less strongly to relaxed myofibrils than to S1. We are investigating the steps involved in the release of ADP but because of the large K_d with myofibrils and parasite enzyme activities this is difficult.

Relevance of Studies on Dispersed Molecules and Myofibrils to Muscle Contraction. An important conclusion from our results, both here and in Houadjeto et al. (1991, 1992), is that kinetically the ATPase sites in myofibrils (relaxed or activated) behave as though they were in solution. In particular, the organization of the myosin heads into the myofibrillar lattice does not seem to affect the initial binding of ATP: transient lag phases were not detected in any of the ATP chase experiments and the second-order binding constant for ATP with myofibrils and S1 are indistinguishable. Further, the overall k_{cat}'s for relaxed myofibrils and S1 and their energies of activation are very similar.

From these similarities we conclude that it is reasonable to extrapolate from studies on dispersed molecules (e.g., as with S1 and acto-S1) to those with the organized myofibrils. Does this mean that we can extrapolate further to the contracting muscle? Any such extrapolation must be done with caution. First, it is possible that the mere skinning of muscle fibers alters their ATPase activity. Second, in muscle fibers mechanical factors such as stress or strain may affect the kinetics of ATP hydrolysis. These factors are largely absent in acto-S1 or myofibrils. For a discussion of these problems, see Huxley (1980, 1988) and Goldman (1987).

ACKNOWLEDGMENT

We are grateful to Dr. M. Geeves for stimulating discussions.

REFERENCES

Bagshaw, C. R., & Trentham, D. R. (1973) *Biochem. J. 133*, 323–328.
Barman, T. E., & Travers, F. (1985) *Methods Biochem. Anal. 31*, 1–59.
Biosca, J. A., Barman, T. E., & Travers, F. (1984) *Biochemistry 23*, 2428–2436.
Biosca, J. A., Travers, F., Barman, T. E., Bertrand, R., Audemard, E., & Kassab, R. (1985) *Biochemistry 24*, 3814–3820.
Biosca, J. A., Greene, L. E., & Eisenberg, E. (1988) *J. Biol. Chem. 263*, 14231–14235.
Bowater, R., Webb, M. R., & Ferenczi, M. A. (1989) *J. Biol. Chem. 264*, 7193–7201.
Bremel, R. D., & Weber, A. (1972) *Nature (London) New Biol. 238*, 97–101.
Brenner, B., Schoenberg, M., Chalovich, J. M., Greene, L. A., & Eisenberg, E. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 7288–7291.
Cooke, R. (1986) *CRC Crit. Rev. Biochem. 21*, 53–118.
Eisenberg, E., & Hill, T. L. (1985) *Science 227*, 999–1006.
Evans, J. A. (1988) *Biophys. J. 53*, 191a.
Ferenczi, M. A. (1986) *Biophys. J. 50*, 471–477.
Geeves, M. A. (1991) *Biochem. J. 274*, 1–14.
Goldman, Y. (1987) *Annu. Rev. Physiol. 49*, 637–654.
Goodno, C. C., Wall, C. M., & Perry, S. V. (1978) *Biochem. J. 175*, 813–821.
Goody, R. S., Hofmann, W., & Mannherz, G. H. (1977) *Eur. J. Biochem. 78*, 317–324.

Hibberd, M. G., & Trentham, D. R. (1986) *Annu. Rev. Biophys. Chem. 15*, 119–161.
Hibberd, M. G., Webb, M. R., Goldman, Y. E., & Trentham, D. R. (1985) *J. Biol. Chem. 260*, 3496–3500.
Homsher, E., & Millar, N. C. (1990) *Annu. Rev. Physiol. 52*, 875–896.
Houadjeto, M., Barman, T., & Travers, F. (1991) *FEBS Lett. 281*, 105–107.
Houadjeto, M., Travers, F., & Barman, T. (1992) *Biochemistry 31*, 1564–1569.
Huxley, A. F. (1980) *Reflections on Muscle*, Liverpool University Press, Liverpool, England.
Huxley, A. F. (1988) *Annu. Rev. Physiol. 50*, 1–16.
Knight, P. J., & Trinick, J. A. (1982) *Methods Enzymol. 85B*, 9–15.
Miyata, M., Arata, T., & Inoue, A. (1989) *J. Biochem. (Tokyo) 105*, 271–274.
Moss, R. L., & Haworth, R. A. (1984) *Biophys. J. 45*, 733–742.
Payne, M. R., & Rudnick, S. E. (1989) *Trends Biochem. Sci. 14*, 357–360.
Rosenfeld, S. S., & Taylor, E. W. (1984) *J. Biol. Chem. 259*, 11908–11919.
Rosenfeld, S. S. & Taylor, E. W. (1987) *J. Biol. Chem. 262*, 9984–9993.
Shukla, K. K., Levy, H. M., Ramirez, F., Marecek, J. F., Mc Keever, B., & Margossian, S. S. (1983) *Biochemistry 22*, 4822–4830.
Sleep, J. A. (1981) *Biochemistry 20*, 5043–5051.
Sleep, J. A., & Hutton, R. L. (1978) *Biochemistry 17*, 5423–5430.
Sutoh, K., & Harrington, W. F. (1977) *Biochemistry 16*, 2441–2449.
Taylor, E. W. (1979) *CRC. Crit. Rev. Biochem. 10*, 102–164.
Taylor, E. W. (1990) *Biophys. J. 57*, 336a.
Tesi, C., Bachouchi, N., Barman, T., & Travers, F. (1989) *Biochimie 71*, 363–372.
Tesi, C., Barman, T., & Travers, F. (1990) *FEBS Lett. 260*, 229–232.
Tesi, C., Kitagishi, K., Travers, F., & Barman, T. (1991) *Biochemistry 30*, 4061–4067.
Trentham, D. R., Eccleston, J. F., & Bagshaw, C. R. (1976) *Q. Rev. Biophys. 9*, 217–281.
White, H. D. (1985) *J. Biol. Chem. 260*, 982–986.
Yates, L., & Greaser, M. L. (1983) *J. Mol. Biol. 168*, 123–141.