Time resolved measurements show that phosphate release is the rate limiting step on myofibrillar ATPases

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Abstract The myofibril is a good model to study the ATPase of the muscle fibre. When myofibrillar ATPase reaction mixtures are quenched in acid, there is a burst of P_i formation, due to AM·ADP·P_i or P_i, as shown in the scheme: AM + ATP ↔ A·M·ATP ↔ AM·ADP·Pi ↔ AM·ADP + P_i ↔ AM + ADP. Therefore, in the steady state, either AM·ADP·P_i or AM·ADP or both predominate. To determine which, we studied the reaction using a P_i binding protein (from *E. coli*) labeled with a fluorophore such that it is specific and sensitive to free P_i [Brune, M. et al. (1994) Biochemistry 33, 8262–8271]. We show that the P_i bursts with myofibrillar ATPases (calcium-activated or not, or crosslinked) are due entirely to protein bound P_i. Thus, with myofibrillar ATPases the AM·ADP·P_i state predominates.

Key words: Muscle contraction; Myofibril (rabbit psoas); ATPase-Mg^{2+}; P_i binding protein (*E. coli*); Enzyme kinetics; Rate limiting step

1. Introduction

A central problem in muscle contraction is to relate the mechanical events with the chemical steps of the myosin head ATPase. The intermediates on the ATPase pathway interact in different ways with the thin filament (actin) and it is this interaction that is responsible for the contractile process. Therefore, identifying the predominant intermediates on this pathway and the rates of their interconversion is not merely a kinetic nicety but an essential step towards understanding muscle contraction.

A good model for the muscle fibre is the myofibril. Myofibrils are the functional contractile units of muscle fibres yet they are small enough for study by transient kinetics techniques, so that detailed kinetic measurements are possible. With myofibrils, force measurements have been carried out [1,2] and their chemical kinetics have been studied in detail (e.g. [3–7]). For the interpretation of the kinetics we consider a condensed version of the mechanism, adapted to the studies presented here and shown in Scheme 1. In the scheme, M represents myosin heads and A actin and the AM·ATP and AM·ADP·P_i states are shown as associated, although actin may readily dissociate from these intermediates [8] but we have little information on these processes with myofibrils. Further, an important feature of the scheme is that ATP is bound essentially irreversibly [3–5]:

$$\mathrm{AM + ATP} \rightleftharpoons \mathrm{AM\cdot ATP} \rightleftharpoons \mathrm{AM\cdot ADP\cdot P_i}$$

$$\mathrm{AM\cdot ADP} \xrightarrow{k_3} \mathrm{P_i} \qquad \mathrm{AM\cdot ADP} \xrightarrow{k_4} \mathrm{AM + ADP}$$

Scheme 1

A key method for identifying myosin ATPase intermediates is to carry out 'P_i burst' experiments [9]. In these, myofibrils plus ATP reaction mixtures, milliseconds to several seconds old, are quenched in acid and the P_i determined. According to Scheme 1, this P_i can be due to myosin bound P_i (AM·ADP·P_i) that decomposes in the acid quench as well as due to free P_i. Here we are particularly interested in the products' release steps, defined by k_3 for P_i and k_4 for ADP.

When the P_i burst method was applied to the myofibrillar ATPases (relaxed or calcium-activated), there were large P_i burst transient phases [4,7] but the origin of this P_i was not determined. Myofibrils prevented from shortening by chemical crosslinking and hence a model for isometric fibres also gave a large P_i burst [5]. The problem, then, is to determine if the P_i bursts with myofibrils are due to bound or free P_i: in the first case the predominant steady state intermediate would be AM·ADP·P_i or M·ADP·P_i if the actin is dissociated, in the second case AM·ADP.

Recently, Brune et al. [10] described a method that is specific and sensitive for free P_i with which it is possible to make real time measurements of P_i production in the millisecond time range and sub-micromolar concentration range. The method is based upon a P_i probe that is a mutant of the *E. coli* phosphate binding protein (PBP) specifically labeled at an introduced cysteine with a fluorophore, such that the labeled protein has fluorescence sensitive to P_i binding. Here we applied the PBP method to the myofibrillar ATPases and show that their P_i bursts are due entirely to protein bound P_i. To increase the time resolution, measurements were done at 4°C but it appears that at 20°C, too, the P_i bursts with calcium-activated and crosslinked myofibrils are due to protein-bound P_i. For convenience sake, we term myofibrils in the absence of Ca, 'relaxed' and in the presence of Ca, 'activated' [4].

2. Materials and methods

2.1. Materials

Myofibrils were obtained from rabbit psoas muscle and cross-linked as in [5]. The concentration of myosin heads was measured as in [5]. All measurements were made on at least two separate myofibrillar preparations from different rabbits.

The free P_i probe was the A197C mutant of the *E. coli* phosphate

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Abbreviations: PBP, phosphate binding protein; MDCC, *N*-[2-(1-maleimidyl)ethyl]-7-diethylaminocoumarin-3-carboxamid e; S1, myosin subfragment 1.

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binding protein (PBP) that had been labeled specifically at the introduced cysteine with *N*-[2-(1-maleimidyl)ethyl]-7-diethylaminocoumarin-3-carboxamide (MDCC), a coumarin fluorophore [10]. [γ-^{32}P]ATP was from Amersham International. 'Bacterial' purine nucleoside phosphorylase and 7-methylguanosine were from Sigma.

2.2. Experimental conditions

The buffer was 0.1 M K-acetate, 5 mM KCl and 50 mM Tris-acetate, pH 7.4. With activated myofibrils, the buffer included 2 mM Mg-acetate and 0.1 mM $CaCl_2$ and with relaxed myofibrils 5 mM Mg-acetate and 5 mM EGTA. Although crosslinked myofibrils are rigor activated, their ATPase activities were measured in the presence of 0.1 mM $CaCl_2$. The experiments were carried out at 4 or 20°C, as indicated in the figure legends.

2.3. Total P_i assays: rapid flow quench experiments

P_i burst experiments were carried out using home-built thermostatically controlled rapid flow quench apparatuses [4]. Two were used, depending on the time scale required (see section 3). Myofibrils and [γ-^{32}P]ATP were mixed in the rapid flow quench apparatus, the reaction mixture allowed to age (see figures), then quenched in 22% TCA + 1 mM KH_2PO_4 and the $^{32}P_i$ determined [11]. This P_i is the sum of free P_i and bound P_i from the acid decomposition of AM·ADP·P_i (Scheme 1).

2.4. Free P_i assays: stopped flow experiments

These were carried out in a stopped flow apparatus built in this laboratory [12]. Excitation at 436 nm was with a mercury lamp and there was a J474a (from Specivex) or 455nm (from Oriel) cut-off filter on the emission. Both enzyme and substrate syringes contained 10–20 μM phosphate binding protein (MDCC-PBP). To ensure the removal of trace amounts of P_i contamination present at the start of the measurement, the syringes also contained a P_i mop system [10], i.e. purine nucleoside phosphorylase (0.1 enzyme unit per ml) plus 7-methylguanosine (0.2 mM).

Caution was taken to minimize P_i contamination by the use of plasticware, highly purified water, etc. The stopped flow apparatus was incubated for 10 min with the mop system before the experiments. No significant amount of contaminating P_i was found in the buffers.

The fluorescent signal emitted by the MDCC-PBP upon the binding of the free P_i produced by the myofibrils was calibrated by mixing a known amount of P_i (typically 1 μM) to a solution containing 10 μM MDCC-PBP, 1 or 0.5 μM myofibrils and the P_i mop system. Thus, we obtained a fast increase in the fluorescence signal due to the binding of P_i to MDCC-PBP, and then a slow (100–200 s) decrease due to the removal of the P_i from the MDCC-PBP by the mop system. It was the amplitude of this decrease in fluorescence that we used to calibrate our experiments.

3. Results

3.1. Calcium-activated myofibrils: steady state time courses

A typical P_i burst experiment with activated myofibrils at 4°C is shown in Fig. 1a. There are three phases: a P_i burst of amplitude 0.83 (±0.08) mol P_i/mol myosin head (kinetics not obtained with the apparatus used), a rapid steady state (k_F) and then a deceleration at about 3 s (±0.3) to a slow steady state (k_S). We have already interpreted these phases [4]. The initial burst is during the first turnover, the rapid steady state is subsequent turnovers while the myofibrils are contracting, and the slow rate is following over-contraction of the myofibrils. The phase that interests us here is the initial burst. Its amplitude is close to the ATPase site concentration of the myofibrils [4,7] and it is due to the formation of AM·ADP·P_i and/or free P_i (Scheme 1). This high amplitude shows that either or both AM·ADP·P_i and AM·ADP are the predominant intermediates on the myofibrillar ATPase. To determine which intermediate predominates, we studied the ATPase in a stopped flow apparatus using the specific probe for free P_i, MDCC-PBP.

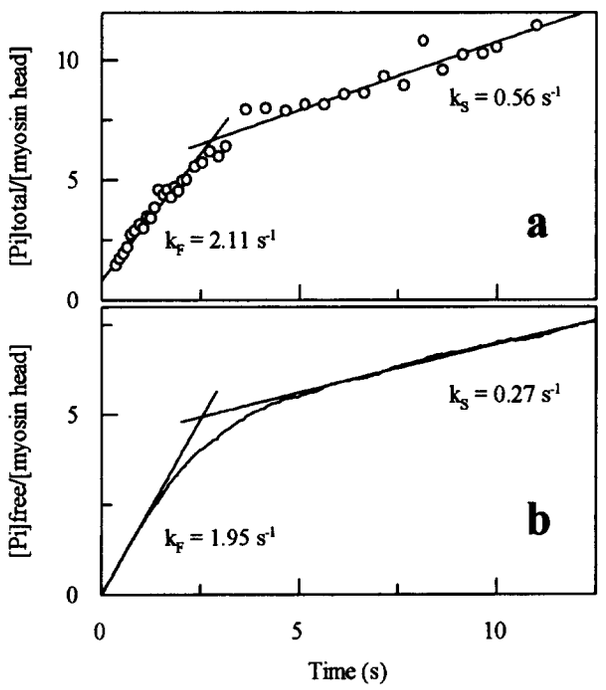

Fig. 1. Time courses for the ATPase of calcium-activated myofibrils by flow quench (total P_i, a) and stopped flow (free P_i, b) at 4°C. The reaction mixtures were 30 μM [γ-^{32}P]ATP + 2.5 μM in myosin heads (flow quench) and 30 μM ATP + 1 μM in myosin heads (stopped flow).

A typical time course for free P_i production is shown in Fig. 1b. There were only two phases: a rapid steady state (1.95 ± 0.15 s^{-1}) followed by a break at about 3 s (±0.3) to 0.27 ± 0.03 s^{-1}. It is noteworthy that by this method an initial P_i burst could not be detected.

The rapid steady state and its duration were nearly identical to those found by the flow quench method. The final slow steady state rate was significantly lower than that found by acid quenching; this is probably due to the P_i mop system competing with the MDCC-PBP for P_i. The lack of a burst of free P_i suggests strongly that the P_i burst with activated myofibrillar ATPase is due to protein bound P_i, i.e. that the predominant intermediate is AM·ADP·P_i rather than AM·ADP. To confirm this, we must obtain the kinetics of the P_i burst in the millisecond time range.

3.2. Calcium-activated myofibrils: transient phases of AM·ADP·P_i and P_i production

We obtained the kinetics of the P_i burst by using a rapid flow quench apparatus that samples reaction mixtures in the millisecond time range (Fig. 2, curve a). The burst had an amplitude of 0.84 ± 0.08 mol P_i/mol myosin head (very similar to that in the steady state experiments (Fig. 1)) and a rate constant of 20 ± 2 s^{-1}. That this burst is due to protein bound P_i was confirmed by the PBP method by which there was no P_i burst but instead a large transient lag phase in free P_i production (Fig. 2, curve b). Taken together, these results show that under our experimental conditions the predominant intermediate on activated myofibrillar ATPase is AM·ADP·P_i and that the P_i release step is rate limiting.

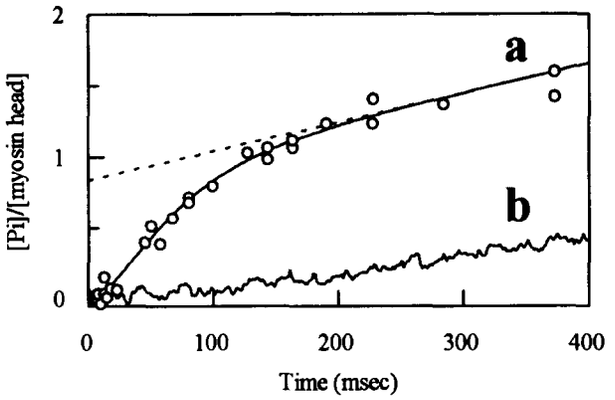

Fig. 2. Initial time courses for P_i burst with activated myofibrils by flow quench (a) and stopped flow (b) at 4°C. The reaction mixtures were 50 μM [γ-^{32}P]ATP + 10 μM in myosin heads (flow quench) and 30 μM ATP + 1 μM myosin heads (stopped flow).

3.3. Crosslinked myofibrils

In P_i burst experiments with cross-linked myofibrils there were only two phases: a large P_i burst followed by a rapid steady state rate, similar to that with native myofibrils. There was no deceleration of the steady state rate because these myofibrils cannot contract [5]. Here by the PBP method, a P_i burst could not be detected (result not illustrated). Therefore, with crosslinked myofibrils, too, AM·ADP·P_i appears to be the predominant steady state intermediate.

3.4. Relaxed myofibrils

A typical P_i burst experiment with relaxed myofibrils is shown in Fig. 3 (curve a). There was a transient burst phase of 0.92 ± 0.09 mol P_i per mol myosin head followed by a steady state rate of 0.019 ± 0.002 s^{-1}.

The time course for free P_i with relaxed myofibrils is shown in Fig. 3 (curve b). There was an insignificant burst of P_i (see below); the steady state rate (0.020 ± 0.002 s^{-1}) was very similar to that found in the P_i burst experiment.

In both of the time courses, there are 'minibursts' of P_i: in the flow quench experiment it immediately follows the initial P_i burst phase, in the stopped flow experiments it was an initial phase. These small phases are almost certainly not due to transient intermediates, as their kinetics are much too slow ($t_{1/2}$ about 2 s). Although carried out under multi-turnover conditions, they are more likely due to rigor activation caused by not all of the myosin heads in the myofibrils binding ATP at the same time [13]. This idea is supported by the increase in amplitude of this rapid phase to the equivalent of several turnovers at very low ATP concentrations or by increasing the temperature from 4 to 20°C. We did not investigate these &lsqou;minibursts' any further here.

Taken together, these experiments show that with relaxed myofibrils, too, the complex AM·ADP·P_i predominates in the steady state and that there is little accumulation of AM·ADP (Scheme 1).

3.5. Experiments at 20° C

Fibre mechanics are in general carried out at 20°C and it was important to confirm our results at this higher temperature. In Fig. 4 are illustrated two P_i progress curves with activated myofibrils: curve (a) obtained by the rapid flow quench method (therefore total P_i) and curve (b) by the PBP method (free P_i only). Because of the high steady state ATPase activity of activated myofibrils, measurements at 20°C are more difficult than at 4°C. Nevertheless, it appears that at 20°C, too, there was a significant burst of AM·ADP·P_i but not of free P_i – indeed in the PBP experiment there was a lag in free P_i production. A similar result was obtained with crosslinked myofibrils (not illustrated). Therefore, as at 4°C, the AM·ADP·P_i complex predominates in the steady state and AM·ADP does not accumulate with either native or crosslinked myofibrils. Because of problems with rigor activation, it was difficult to obtain convincing P_i burst data with relaxed myofibrils at 20°C.

4. Discussion

Although there have been several studies of the myofibrillar ATPases [3–7], these were confined to the early steps of the pathway, i.e. the binding and hydrolysis of ATP (steps 1 and 2 in Scheme 1). The later release of products have been intractable to study. With myosin ATPase, the products are released slowly, with P_i coming off before ADP [14], and it seems reasonable to suppose that a two-step release also applies to the myofibrillar ATPases. The difficulty in obtaining information on the kinetics of the release of the products with myofibrils has been frustrating as it appears that these kinetics are involved closely with the contractile process.

The P_i release step is of particular interest as it is thought to be closely linked with force generation in fibres (e.g. [15]). With myofibrils, large P_i bursts are obtained by the chemical quenching method but this method does not distinguish between bound and free P_i. The availability of a method that is at once specific and sensitive for P_i has made this distinction possible [10]. Here we applied this PBP method to the ATPases of myofibrils under three conditions: relaxed (without Ca), isotonic (Ca activated, i.e. shortening under near zero load) and isometric (i.e. myofibrils prevented from shortening by chemical crosslinking). Our experiments were carried out in a buffer of near physiological ionic strength and at 4 and 20°C. We interpreted our data by Scheme 1. We show that the P_i bursts obtained by the chemical quenching on the three materials are entirely due to bound P_i. Indeed, with activated myofibrils, there was a transient lag phase of free P_i during the time that

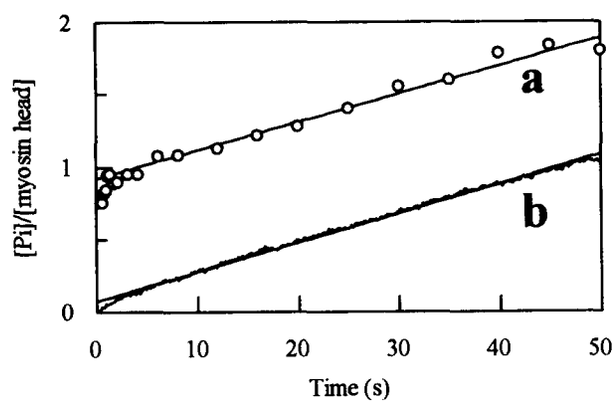

Fig. 3. Time courses for the ATPase of relaxed myofibrils by flow quench (a) and stopped flow (b) at 4°C. The reaction mixtures were 30 μM [γ-^{32}P]ATP + 3 μM in myosin heads (flow quench) and 30 μM ATP + 1 μM in myosin heads (stopped flow).

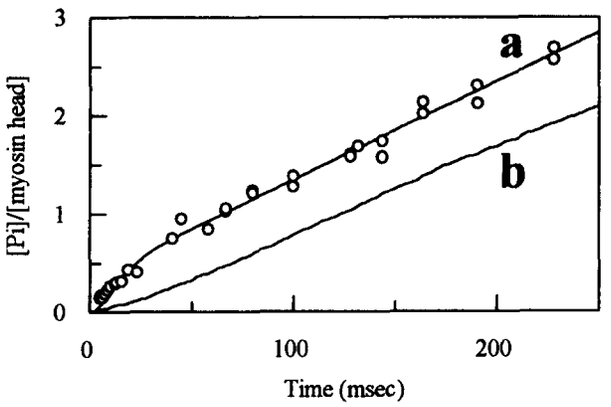

Fig. 4. Initial time courses for P_i burst with activated myofibrils by flow quench (a) and stopped flow (b) at 20°C. The reaction mixtures were 48 μM [γ-^{32}P]ATP + 4.5 μM in myosin heads (flow quench) and 100 μM ATP + 1 μM myosin heads (stopped flow). The curve in (a) is qualitative only and shows that by the flow quench method there is a burst of P_i. The steady state rates in the two experiments are very similar (about 10 s^{-1}).

AM·ADP·P_i increases to the steady state. This lag suggests that the kinetics of the ADP release step are fast, i.e. that the concentration of AM·ADP is low. This situation appears to hold at 20°C as well as 4°C.

We show that with myofibrils under the three conditions, the rate limiting step is the release of P_i, i.e. that the complex AM·ADP·P_i accumulates in the steady state, as is the case with myosin subfragment 1 (S1) ATPase. What is noteworthy, is that with the myofibrils and with S1 the kinetics of the three initial steps are very similar. It is the kinetics of the two step release of products that distinguishes the systems.

With S1 ATPase, the kinetics of the release of P_i (k_3, Scheme 1) and ADP (k_4) are the slowest steps [14]. As $k_3 < k_4$, k_{cat} is governed principally by k_3.

With 'relaxed' myofibrils, too, k_{cat} seems to be governed by k_4. Further, the k_{cat} is very similar to that with S1 [4]. So, with the S1 and relaxed myofibrillar ATPases, the kinetics of the first four steps appear to be very similar. We have not measured k_4 with relaxed myofibrils but it may be much faster than with S1. This possibility rests upon the difference in the overall binding constant of ADP to myofibrils whether relaxed or not (140 μM [16]) and S1 (< 1 μM [14]).

With 'activated' or 'cross-linked' myofibrillar ATPases, k_3, although much faster than with S1 or relaxed myofibrils, remains the rate limiting step. Since there was a large transient lag in free P_i production, it appears that $k_4 \gg k_3$. Therefore, with these myofibrils, the kinetics of the release of both P_i and ADP are much faster than with S1. In contrast, with actoS1 the rate limiting step is early, probably the cleavage itself: there is no burst of P_i either bound or free [10,17–19]. As with the myofibrils, the ADP release with actoS1 is rapid [20].

How do we relate our results, obtained from myofibrils, to current ideas on the disposition of the ATPase states in fibre contraction? Our working hypothesis is that fibres contracting rapidly under zero load are modeled by activated myofibrils [3] and isometric fibres by crosslinked myofibrils [5]. Here we have shown that AM·ADP·P_i is the major accumulated state for all myofibrillar conditions and we now consider some possible implications of this in the muscle contraction mechanism.

It is generally believed that the chemical states that are involved in active force generation are strong actin binding states containing ADP, especially AM·ADP [20]. From mechanical studies using caged P_i it has been shown that during isometric contraction of fibres both AM·ADP·P_i and AM·ADP contribute significantly to force [21]. From our results, especially using crosslinked myofibrils, it appears that during contraction the concentration of AM·ADP is very low so that if this were the sole force generating state, the efficiency would be low. This in turn implies that the highly populated AM·ADP·P_i state may well contribute to force generation, following the necessary transition from a weak to strong binding state, as previously suggested [8,15]

We conclude with a word of caution. In most kinetic studies on muscle systems (from fibres to actoS1) the myosin head ATPase is triggered by ATP rather than calcium, although the latter is normal in real life and the method of activation may affect the mechanism at early stages. To determine the effect of calcium triggering upon the kinetics of the ATPase, further experiments are necessary.

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