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Cryoenzymic Studies on Actomyosin ATPase: Kinetic Evidence for Communication between the Actin and ATP Sites on Myosin†

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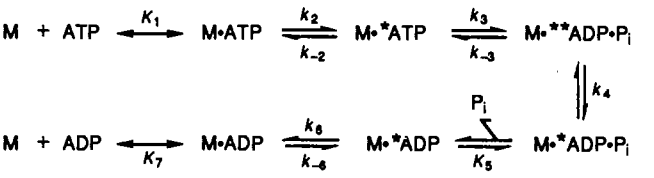
Received June 29, 1990; Revised Manuscript Received October 31, 1990

ABSTRACT: The post-ATP binding steps of myosin subfragment 1 (S1) and actomyosin subfragment 1 (actoS1) ATPases were studied at −15 °C with 40% ethylene glycol as antifreeze. The cleavage and release of P_i steps were studied by the rapid-flow quench method and the interaction of actin with S1 plus ATP by light scattering in a stopped-flow apparatus. At −15 °C, the interaction of actin with S1 remains tight, and the K_m for the activation of S1 ATPase is very small (0.3 μM). The chemical data were interpreted by E + ATP → E*·ATP ↔ E**·ADP·P_i → E*·ADP → products, where E is S1 or actoS1. In P_i burst experiments with S1, there was a large P_i burst of free P_i, but E**·ADP·P_i could not be detected. Here the predominant complex in the seconds time range is E*·ATP and in the steady-state E*·ADP. With actoS1, there was a small P_i burst of E**·ADP·P_i, evidence that the cleavage steps for S1 and actoS1 are different. From the stopped-flow experiments, the dissociation of actoS1 by ATP was complete, even at actin concentrations 60× its K_m. Further, no interaction of actin with the key intermediate M*·ATP could be detected. Therefore, at −15 °C, actoS1 ATPase occurs by a dissociative pathway; in particular, the cleavage step appears to occur in the absence of actin. It is proposed that the actin-induced conformation of myosin (M^+) is very sensitive to the temperature: under "normal" conditions, it isomerizes rapidly to the M* conformation which would explain the near-identity of the cleavage step of S1 and actoS1, but at −15 °C, this isomerization is slow, so the chemical step occurs with the M^+ conformation which could explain the difference of the chemical steps. A reaction scheme for actoS1 is proposed which is based on this and already published data.

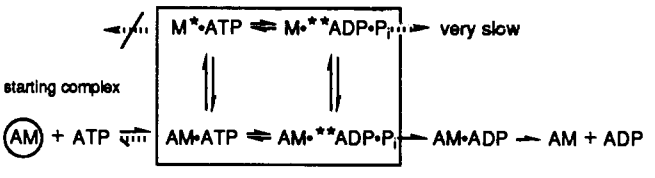
Muscle contraction depends on the cyclic interaction of actin and myosin, the energy for which is supplied by the hydrolysis of adenosine 5′-triphosphate (ATP)[1] by the myosin heads. It is thought that movement is controlled by actin interacting in different ways with the different intermediates on the myosin ATPase reaction pathway.

The kinetic processes of organized systems are difficult to study directly. Most work on actomyosin has been confined to solution studies using purified myosin—or more often its proteolytic fragments subfragment 1 (S1) or heavy meromyosin (HMM)—and actin [e.g., see Taylor (1979), Hibberd and Trentham (1986), and Cooke (1986)]. Further, experimental protocols have been dictated more by the physicochemical properties of the individual proteins, such as their solubilities and interactions, than by the conditions prevailing in vivo. Certainly a detailed knowledge of the physicochemical properties of the several proteins making up muscle is indispensable toward a full understanding of muscle contraction.

Scheme I



Scheme II



However, any extrapolation of this knowledge to the interpretation of phenomenon observed with, e.g., muscle fibers must be done selectively and with caution.

It is generally agreed that for myosin alone in solution the ATPase pathway is described by the Bagshaw–Trentham

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

0006-2960/91/0430-4061$02.50/0

scheme (Trentham et al., 1976) (Scheme I) where M represents myosin. Different protein conformations are shown by asterisks. There are two important features of this scheme. First, the ATP is bound essentially irreversibly (i.e., $k_{-2} \ll k_{cat}$; Goody et al., 1977) to give the key intermediate M*·ATP (Geeves & Trentham, 1982). This process appears to be independent of the experimental conditions. Second, there is a clear-cut rate-limiting step the identity of which depends upon the temperature: above 5 °C, it is k_4, below k_6 (Malik & Martonosi, 1972).

For a full understanding of muscle contraction, the details of the interaction of actin with the various intermediates of myosin ATPase must be elucitated. Rosenfeld and Taylor (1984) proposed that much of the published data for actoS1 can be accommodated by a simple working model (Scheme II).

From the involvement of four key intermediates (enclosed within the box), this scheme is termed the "four state model" for actomyosin. It must be emphasized that in their studies most workers mix actoS1 (starting complex) with ATP and then observe [a possible cause for concern here is that one starts with a material—the rigor complex—that has only a fleeting existence in the working muscle, e.g., Cooke (1986)].

However, this working model does not take account of the work of Biosca et al. (1984a), namely, that the S1 freshly released from actoS1 by ATP is different from that of S1 alone (the M^+ conformation). This conformation has been confirmed by Millar and Geeves (1988) and Tesi et al. (1990b).

Here our purpose was to investigate further the role of the M^+ conformation on actomyosin ATPase. This conformation is of particular interest as it may be a manifestation of the transmission mechanism of Morales and his colleagues (Morales & Botts, 1979; Botts et al., 1989). According to this, whereas the actin and ATP sites on myosin are well separated, there is transmission of information between them.

One way to obtain physicochemical and mechanistic information on a system is to perturb it. Temperature and solvent perturbation (cryoenzymology) has already been used with myosin (Bechet et al., 1979; Biosca et al., 1984a) and with actomyosin ATPases (Trentham, 1977; Travers & Hillaire, 1979; Millar & Geeves, 1983; Biosca et al., 1984b; Geeves et al., 1986; Tesi et al., 1990b). In these studies, 40% ethylene glycol was used as antifreeze.

We present here a continuation of our previous work on the initial binding of ATP to S1 and actoS1 which was carried out at −15 °C in 40% ethylene glycol (Tesi et al., 1990b). We show, first, that the interaction of actin with myosin remains tight and that the K_m for the activation of the ATPase of S1 by actin is very small (0.3 μM). This makes it easy to work at saturation in actin. However, we could not detect any interaction of actin with M*·ATP, even at concentrations of actin 60 times its K_m. Second, it appears that at −15 °C actoS1 ATPase occurs by a wholly dissociative pathway and yet the kinetics of the chemical step seem to be different from those with S1 alone. Finally, whereas the degree of saturation of actoS1 in S1 affects the kinetics of binding of ATP (Tesi et al., 1990b), it does not affect the kinetics of the chemical and release of P_i steps. We propose a reaction scheme for actoS1 in ethylene glycol in a low ionic strength buffer which takes into account communication between the actin and ATPase sites of the S1.

Materials and Methods

Proteins and Reagents. Myosin and S1 were prepared as in Tesi et al. (1990) and actin as in Spudich and Watts (1971). [γ-^{32}P]ATP was from Amersham International. D-3-Glyceraldehyde-3-phosphate dehydrogenase, phosphoglycerate kinase, D-glyceraldehyde 3-phosphate, NAD, and ADP were from Bœhringer-Mannheim.

Experimental Conditions. Experiments at −15 °C were carried out with 40% ethylene glycol as the antifreeze in 5 mM Tris, 5 mM KCl, 2 mM magnesium acetate, and 1 mM DTT, adjusted to pH 8 with acetic acid. Air bubbles were removed by centrifugation at low speed in a desk centrifuge for 1 min.

Rapid-Flow Quench Experiments. Two types of experiments were carried out. In *ATP chase experiments*, one obtains the kinetics of the tight binding of ATP to S1 or actoS1 with $k = k_2[ATP]/([ATP] + K_1)$ (Trentham et al., 1976; Barman & Travers, 1985). S1 or actoS1 plus [γ-^{32}P]ATP reaction mixtures are first quenched in unlabeled ATP and then in acid, and the [^{32}P]P_i is determined (Reimann & Umfleet, 1978). In *P_i burst experiments*, reaction mixtures are quenched directly in acid and the [^{32}P]P_i determined. Both types of experiments were carried out in thermostatically controlled rapid-flow quench apparatuses constructed in this laboratory (Barman & Travers, 1985).

Stopped-Flow Experiments. These were carried out in an apparatus adapted for work at low temperatures constructed in this laboratory (Markley et al., 1981). To allow for successive reactions, a thermostatically controlled premixer replaces the enzyme reservoir. The dead time at −15 °C for the first mixer is 20 s and the second 5 ms. In certain experiments, it was necessary to determine accurately amplitudes, and these were carried out in a stopped-flow apparatus fitted to an Aminco DW2 spectrophotometer (Travers & Barman, 1980). The dead time of this apparatus is 5 ms.

Treatment of Data. The kinetic data were fitted by using a modified version of the KINFIT program of Knach and Röhm (1981) on an Apple IIe computer. The data from the stopped-flow experiments were stored in a Sord Mark III computer and transferred to the Apple for treatment.

Table I: Effect of Experimental Conditions upon the K_m for Actin in S1 ATPase Obtained from Steady-State Experiments[a]

conditions	K_m (μM)
5 mM Tris, water, 15 °C	18
5 mM Tris, 40% ethylene glycol, 15 °C	5.5
10 mM Tris, 40% ethylene glycol, 15 °C	17.5
20 mM Tris, 40% ethylene glycol, 15 °C	20
30 mM Tris, 40% ethylene glycol, 15 °C	35
50 mM Tris, 40% ethylene glycol, 15 °C	>50
5 mM Tris, 40% ethylene glycol, −15 °C	0.3[b]

[a] The other conditions were 5 mM KCl, 2 mM magnesium acetate, and pH 8 (acetic acid) with actin unsaturated in S1. [b] From ATP chase experiments at low concentrations of actin and S1, from Table II.

Results

Effect of Experimental Conditions on the Steady-State Parameters of ActoS1 ATPase. The effect of actin in activating S1 ATPase appears to follow a hyperbolic law: from the dependence of the steady state upon the actin concentration, one obtains a K_m for actin and a k_{cat} (i.e., the steady state at saturating actin). The effect of the experimental conditions on the K_m is given in Table I.

The effect of increasing the Tris concentration on increasing the K_m is as expected from processes that are sensitive to the ionic strength. What is noteworthy is that 40% ethylene glycol at 15 °C reduces the K_m by about 3 and by lowering the temperature to −15 °C the K_m is reduced further to 0.3 μM. We believe that this is the lowest reported value for the K_m of actin which allowed us to study the actoS1 ATP system at actin concentration $\gg K_m$ for actin.

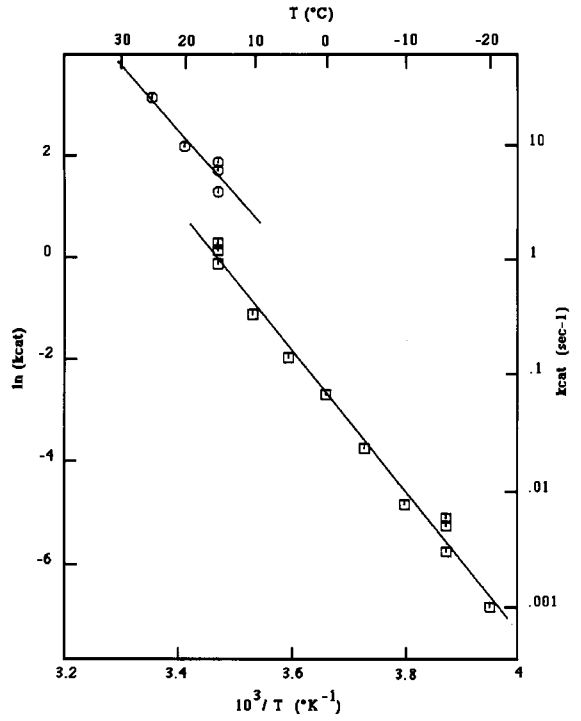

FIGURE 1: Temperature dependencies of the k_{cat} for actoS1 in 40% ethylene glycol (□) and in water (○). For full details, see the text.

The temperature dependencies of the k_{cat} of actoS1 in 40% ethylene glycol and water are given in Figure 1. As found in the preliminary work of Travers and Hillaire (1979), the energy of activation is high ($\Delta H^{\ddagger}$ = 103 ± 21 kJ mol^{-1} in water and 114 ± 4 kJ mol^{-1} in 40% ethylene glycol). Unlike with S1 ATPase, where there is a curvature due to a change in the rate-limiting step (Biosca et al., 1984b), there was no curvature with actoS1 ATPase. The activation energies obtained in the two solvents are similar which suggests that, as with S1 ATPase (Biosca et al., 1984b), 40% ethylene glycol does not affect significantly the overall mechanism of actoS1 ATPase.

Effect of Cryoenzymic Conditions upon the Binding of Actin to S1. The dependence of the kinetics of the binding of S1 to actin (k_{obs}) in the absence of ATP was determined by light scattering in a stopped-flow apparatus. The data were interpreted according to

$$\text{actin} + \text{S1} \underset{k_-}{\overset{k_+}{\rightleftharpoons}} \text{actoS1}$$

with $k_-/k_+ = K_d$.

Thus, by plotting k_{obs} against [actin] at constant [S1], a straight line is obtained with slope k_+ and an intercept on the k_{obs} axis of k_-. Here, $k_+ = 2.4 \times 10^4$ M^{-1} s^{-1}, and $k_- < 0.15$ s^{-1} from which $K_d < 6$ μM (data not illustrated).

A more sensitive way of obtaining K_d is to carry out ATP chase experiments at low concentrations of actin and S1. This is because the second-order binding constants for the binding of ATP to S1 and actoS1 differ by the large factor of 25 (Tesi et al., 1990b). Thus in experiments with actoS1 any free S1 should manifest itself by giving biphasic ATP chase curves.

A typical ATP chase curve at low concentrations of actin, S1, and ATP is illustrated in Figure 2. There was a transient burst phase whose amplitude gives the ATPase site concentration and kinetics those of the tight binding of ATP. This was followed by the steady-state rate which divided by the active-site concentration gives k_{cat} (Barman & Travers, 1985). It is noteworthy that the fast kinetics were cleanly first order and there was no indication of any biphasicity. The kinetic

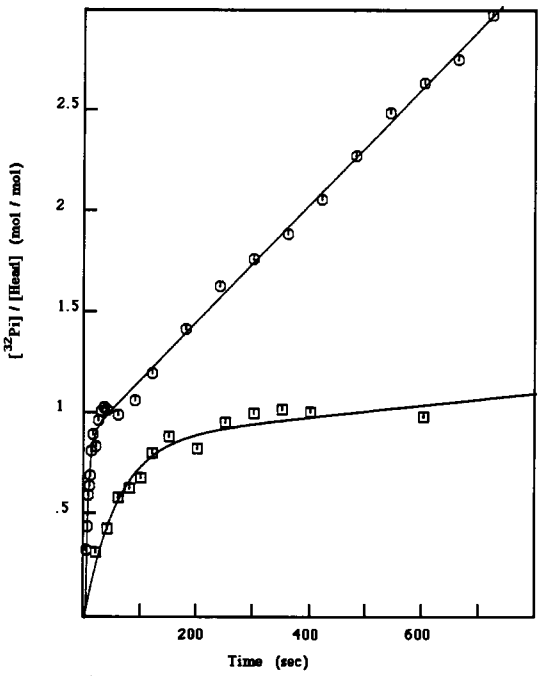

FIGURE 2: ATP chase experiment at a low concentration of actoS1. The reaction mixture was 0.125 μM S1, 0.25 μM actin, and 1.5 μM ATP. The curve was fitted to k = 0.172 s^{-1}, an amplitude of 0.87 mol of ATP bound/mol of S1, and a steady state of 2.9 × 10^{-3} s^{-1}. By dividing the steady state by the amplitude, k_{cat} = 3.3 × 10^{-3} s^{-1} [see Barman and Travers (1985) for treatment]. For points (○), the time scale is as indicated; for (□), it is divided by 10.

Table II: Effects of Low Concentrations of Actin upon the Kinetics of the Binding of ATP to S1 (k) and Its ATPase Activity at −15 °C[a]

[actin] (μM)	[S1] (μM)	k (s^{-1})	steady state[b] (×10^{-3} s^{-1})
0	0.5	0.008 (±0.0005)	0.25
0.25	0.125	0.17 (±0.01)	3.3
0.5	0.125	0.17 (±0.01)	3.8
1	0.5	0.14 (±0.02)	4.1
5	0.5	0.15 (±0.01)	5.2

[a] The kinetic constants were obtained from ATP chase experiments (e.g., Figure 2). The concentration of ATP was 1.5 μM. [b] Corrected to the active-site concentrations (Barman & Travers, 1985).

constants obtained from this experiment, and three others at the same concentration of ATP but different S1 and actin concentrations, are summarized in Table II. At 1.5 μM ATP, with S1 alone, k = 0.008 s^{-1}. From the steady-state phase, the K_m for actin and the k_{cat} were obtained (see above).

There are three conclusions from these experiments. First, from the lack of variation of k with the S1 and actin concentrations, the K_d for the interaction of S1 with actin is <0.05 μM. Thus, with a K_d of 0.05 μM at 0.25 μM actin and 0.125 μM S1, the free [S1] is 0.03 μM, which would have been just detectable in the ATP chase experiments. Second, from the dependence of k_{cat} on the actin concentration, the K_m for actin is 0.3 μM, and k_{cat} = 5.8 × 10^{-3} s^{-1}. As with S1 alone, k_{cat} = 2.5 × 10^{-4} s^{-1}; the activating effect of actin at −15 °C (23×) is less than under "normal" conditions (100× or more). Third, since k_{cat} values at 1.5 and 15 μM ATP are very similar (curve at 15 μM ATP not shown), the K_m for ATP <1.5 μM.

Dissociation of ActoS1 by ATP at −15 °C. We already had an indication from our previous stopped-flow experiments that at high concentrations of actin (40 times its K_m) the dissociation appeared to be complete (Tesi et al., 1990b). However, with the instrument used in that work, it was not always easy to measure accurately dissociation amplitudes and to inves-

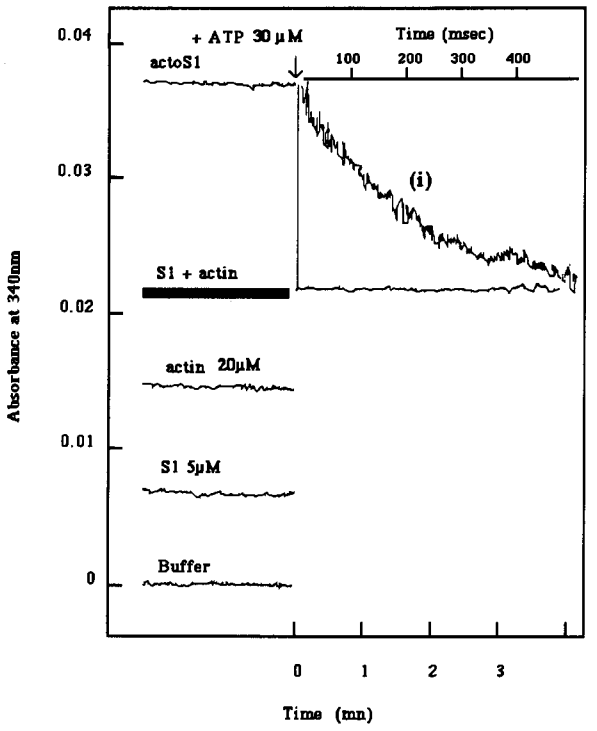

FIGURE 3: Degree of dissociation of actoS1 by ATP. The experiment was carried out in a stopped-flow apparatus fitted to an Aminco DW2 spectrophotometer. One syringe contained 40 μM actin and 10 μM S1 and the other 60 μM ATP. The decrease in turbidity was followed at 340 nm. The insert (i) shows the initial dissociation on an expanded time scale.

Table III: Certain Kinetic Constants for S1 and ActoS1 ATPases at −15 °C[a]

S1		actoS1		
constant	value	constant	saturated	unsaturated
K_1 (μM)	12 (±3)	K'_1 (μM)	14.3 (±3.6)	>300
k_2 (s^{-1})	0.072 (±0.01)	k'_2 (s^{-1})	2.15 (±0.3)	>40
K_3[c]	~0.08	k'_3 (s^{-1})	0.01 (±0.003)	
k_4[c] (s^{-1})	~0.03	k'_{-3} (s^{-1})	0.023 (±0.008)	
k_0[b] (s^{-1})	3×10^{-3}	K'_3	0.43 (±0.05)	
k_6 (s^{-1})	2.5×10^{-4}	k_4 (s^{-1})	0.026 (±0.005)	
k_{cat} (s^{-1})	2.5×10^{-4}	k_{cat} (s^{-1})	5×10^{-3}	

[a] The values for K_1 and k_2 are from Tesi et al. (1990b). For full details, see Materials and Methods. The constants for actoS1 were obtained by using the scheme in Figure 7. [b] k_4K_3. [c] Extrapolated from high-temperature experiments (Biosca et al., 1984b).

tigate the degree of dissociation in more detail: we used a mixing device fitted to an Aminco spectrophotometer. A typical dissociation experiment at 20 μM actin (about 65 times its K_m) is shown in Figure 3.

The accurate determination of the dissociation amplitude was difficult as not only actin but also S1 on its own is turbid [e.g., see Biosca et al. (1983)]. Nevertheless, the experiments were reproducible, and it appears that the dissociation was more than 95% (±5%) complete. The amplitude was independent of the actin concentration. Following dissociation, there was no detectable change in absorption until the ATP had been hydrolyzed (about 20 min), when there was a slow increase (not shown) toward the initial turbidity value. Thus, at −15 °C, it appears that the steady-state complexes on actoS1 ATPase do not contain actin: the system seems to be dissociative, even at actin concentrations $\gg K_m$ for actin. What are the structures of these complexes; do they contain ATP or ADP and P_i? P_i burst experiments can give an answer to this question. However, first we carried out these experiments with S1 alone.

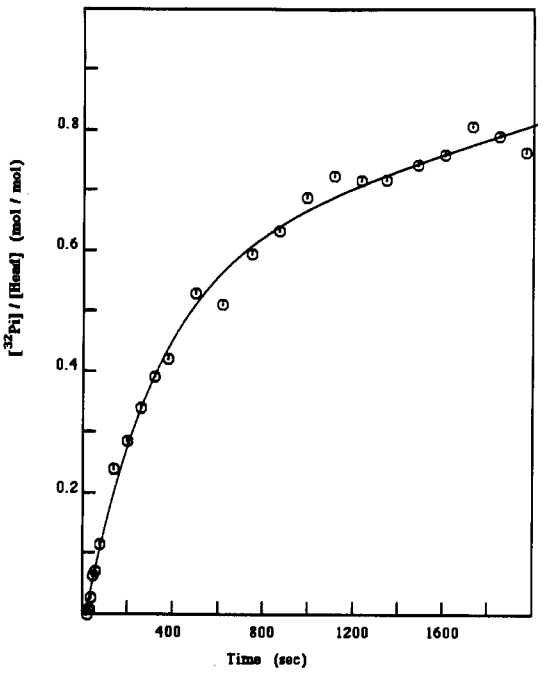

FIGURE 4: Time course for a P_i burst with S1. The reaction mixture was 9.6 μM S1 plus 44 μM [γ-^{32}P]ATP. The data were fitted to an exponential rise (of $k_{obs} = 3.2 \times 10^{-3}$ s^{-1} and amplitude 0.78 mol of P_i/mol of active site) followed by $k_{cat} = 1.6 \times 10^{-4}$ s^{-1}.

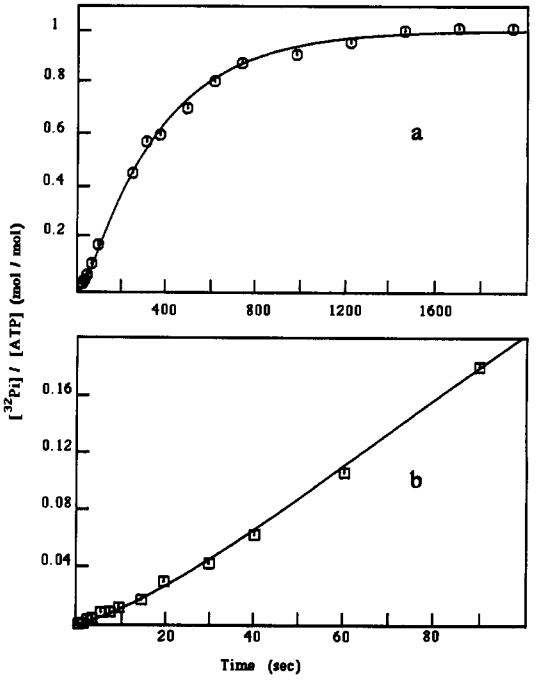

FIGURE 5: Time course for a single-turnover P_i burst experiment with S1. The reaction mixture was 5 μM S1 plus 0.55 μM [γ-^{32}P]ATP. The data were fitted to a transient lag phase ($k = 0.029$ s^{-1}) followed by an exponential rise ($k_0 = 3 \times 10^{-3}$ s^{-1}). (a) Complete time course; (b) initial time course.

P_i Burst Experiments with S1 at −15 °C. The initial binding of ATP to S1 had already been studied at −15 °C and K_1 and k_2 (Scheme I) obtained (see Table III; Tesi et al., 1990b). A typical P_i burst experiment with S1 is illustrated in Figure 4. There was a large P_i burst of amplitude of 0.78 mol of P_i/mol of S1 sites of kinetics $k_{obs} = 3.2 \times 10^{-3}$ s^{-1} which was followed by $k_{cat} = 1.6 \times 10^{-4}$ s^{-1}. The time course of a P_i burst experiment under single-turnover conditions is given in Figure 5. There was a transient lag phase followed by an exponential rise to the complete hydrolysis of ATP. The lag is probably a manifestation of the initial ATP binding kinetics;

it was fitted to 0.029 s^{-1}, which agrees well with the calculated value (0.023 s^{-1}; from the values for K_1 and k_2 in Table III). The exponential rise was fitted to $k_{obs} = 3 \times 10^{-3}$ s^{-1} which is very similar to that obtained from the exponential phase in P_i burst experiments under multi-turnover conditions (Figure 4).

The similarity of the kinetics of the P_i bursts under multi- and single-turnover conditions suggests but does not prove that they are both due to free P_i. This was confirmed by the use of the linked assay system of Trentham et al. (1972) in a stopped-flow apparatus. This is specific for free P_i. Typically, one syringe of the apparatus contained 0.8 mM D-glyceraldehyde-3-phosphate, 2 mM NAD, 1 mM ADP, 0.6 mg mL^{-1} D-3-glyceraldehyde-3-phosphate dehydrogenase, 0.2 mg mL^{-1} phosphoglycerate kinase, and 50 μM ATP. The other syringe contained 5 μM S1. The experimental conditions are given under Materials and Methods. Upon mixing, there was a burst of free P_i of amplitude 0.55 mol of P_i/mol of S1 of kinetics 4.5×10^{-3} s^{-1} which was followed by $k_{cat} = 2 \times 10^{-4}$ s^{-1} (curve not shown). These values are in reasonable agreement with the chemical experiments (Figure 4).

In neither of the chemical experiments could we detect an initial rapid burst phase due to the formation of a myosin ADP·P_i type of complex. Thus, we have no information on the individual constants k_3 and k_{-3} (Scheme I), and, therefore, unlike under "normal" conditions [e.g., see Taylor (1979)], we were unable to obtain direct kinetic information on the cleavage step of S1 but we can come to certain conclusions. From the extrapolation of high-temperature experiments under similar conditions (Biosca et al., 1984b), we suggest that these constants are large with respect to k_2 and k_4. Therefore, step 3 (Scheme I) can be considered as a rapid equilibrium, and the constant observed in single-turnover experiments (Figure 5) is $k_o = k_4K_3$ (Bagshaw & Trentham, 1974). By the extrapolation of high-temperature experiments (Biosca et al., 1984b), we estimate K_3 at about 0.08 and k_4 about 0.03 s^{-1}, from which $k_o = 2.4 \times 10^{-3}$ s^{-1} agrees well with the experimentally obtained k_o (3×10^{-3} s^{-1}; Table III). Thus, we suggest that the low concentration of M**·ADP·P_i is due to $k_3 \ll k_{-3}$. Since $k_{cat} < k_o$, this confirms that at low temperatures the rate-limiting step with S1 is k_6 [see Biosca et al. (1984b) and references cited therein].

We summarize in Table III certain of the kinetic constants for S1 ATPase at −15 °C. Because of the rapidity of the initial binding process (Tesi et al., 1990b), the key intermediate M*·ATP (Geeves & Trentham, 1982) is the predominant species at early times (minutes), but the final steady-state intermediate is M*·ADP.

P_i Burst Experiments with ActoS1 at −15 °C. Typical single- and multi-turnover P_i burst experiments with actoS1 saturated in S1 are given in Figure 6. Identical results were obtained with actoS1 unsaturated in S1 (curves not shown). Therefore, whereas the degree of the saturation of actin with S1 in actoS1 affects the initial ATP binding kinetics, it appears not to change the cleavage and release of P_i steps.

The data in Figure 6 could not be fitted to single exponentials, but good fits were obtained with two: we interpret this as P_i being followed at two successive steps of actoS1 ATPase.

There are two significant differences between these results and those with S1. First, the steady state is 25 times greater with actoS1. Second, with the single-turnover experiment with actoS1 (Figure 6a), the slow exponential could be fitted to a constant (about 4×10^{-3} s^{-1}) very near k_{cat} (5×10^{-3} s^{-1}). With S1, the constants obtained from single-turnover experiments were greater than k_{cat} (Table III). These results show that actin accelerates the release of ADP (k_6, Scheme I), and they suggest that unlike with S1 this step is not rate limiting with actoS1. Because of the rapid k_{cat}, we were unable to test for free P_i by the linked assay of Trentham et al. (1972). We suggest that the first phase is due to protein-bound P_i and the second to free P_i. This is very different from the situation at 15 °C where the P_i burst was insignificant [also at a low ionic strength; see Tesi et al. (1990a) and references cited therein].

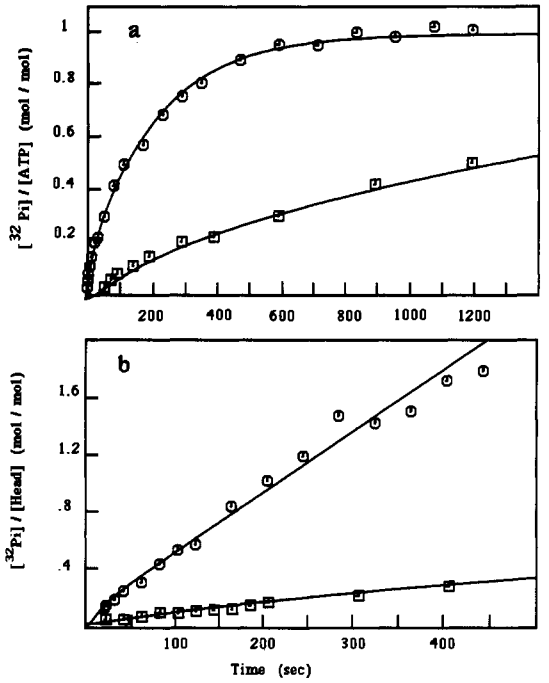

FIGURE 6: Time courses for single- and multi-turnover P_i burst experiments with saturated actoS1. (a) Single-turnover P_i burst; the reaction mixture was 5 μM S1, 5 μM actin, and 0.5 μM [γ-^{32}P]ATP. (b) Multi-turnover P_i burst; the reaction mixture was 5 μM S1, 5 μM actin, and 15 μM [γ-^{32}P]ATP. The curves were obtained by computer fitting using the scheme in Figure 7 and the constants in Table III. For points (○), the time scale is as indicated; for (□), it is divided by 10.

Interaction of Actin with M·ATP.* We made attempts to study directly the interaction of actin with M*·ATP. The experiments were carried out in a stopped-flow apparatus connected to a premixer which allows for the mixing of ATP with S1 before the addition of actin. Thus, to allow for the formation of M*·ATP, S1 and ATP were premixed at −15 °C, and the mixture was allowed to age in one of the syringes of the stopped-flow apparatus (also at −15 °C). After an incubation time of 1 min, the S1 plus ATP mixture was mixed with actin from the other syringe and the increase in turbidity (at 340 nm) determined. Typically, the final reaction mixture was 5 μM S1, 7.5 μM ATP, and 20 μM actin. The increase in turbidity of the final reaction mixture was low. In the absence of ATP, the increase in optical density due to the formation of actoS1 was 0.02. In its presence, less than 15% of this was observed.

In these experiments, the aging time for the S1–ATP mixture was critical. From the chemical work, the interaction of actin with three S1 ATPase intermediates must be considered: remaining free S1, M*·ATP, and M*·ADP. With the constants in Table III, the $t_{0.5}$ for the formation of M*·ATP is about 15 s and that for M*·ADP about 4 min. Thus, after an incubation time of 1 min, the S1 is disposed as follows: free S1, ≈10%, M*·ATP, ≈75%; and M*·ADP, ≈15%. The small increase in turbidity that was observed could be due to the interaction of actin with the remaining free S1 and M*·ADP.

Therefore, any interaction of actin with M*·ATP appears to be weak.

Discussion

On its own, the value of a rate constant for a single step on an enzyme pathway gives little information on the structures of the intermediates involved. However, when the constant changes upon changing some well-defined experimental condition, new structural information may be obtained [e.g., see Gutfreund (1972)]. In particular, if a rate constant on S1 ATPase is changed by actin, it is probable that the S1 has undergone some structural change at its ATPase site. Thus, actin changes its ATP binding kinetics (Biosca et al., 1984a; Tesi et al., 1990b). In addition, the dissociation and tight binding of ATP to actoS1 appear to be separate processes. To explain these results, we reintroduced the actin-induced M^+ conformation of Sleep and Taylor (1976): this is the conformation of S1 that is freshly released by ATP from S1. Recently, Millar and Geeves (1988) came to the same conclusion.

Here we studied the ATP cleavage and release of products steps of S1 and actoS1 at −15 °C. The two materials gave significantly different P_i transients, and we must address ourselves to an explanation for this.

The biphasic P_i burst curve with actoS1 (Figure 6) could be explained by a heterogeneity in the cleavage step. If the cleavage with S1 does not change with actin, then there should be heterogeneity in the cleavage step with S1 too. However, there is little evidence that this is so. Thus, in the P_i burst experiment under single-turnover conditions, there is a lag phase in P_i production (Figure 5). This is a precise experiment as all of the ATP was turned over. It was fitted very well to a simple situation involving only one type of cleavage step: the lag is probably due to the relatively slow buildup of M^+·ATP under these conditions (0.029 s^{-1} obtained; 0.023 s^{-1} calculated from K_1 and k_2 in Table III). There is no evidence that this lag masks a rapid P_i burst transient. If there were such a burst, its amplitude would be less than 0.02 mol of P_i/mol of S1. This is considerably less than the amplitude of the rapid P_i burst obtained with actoS1 [steady-state extrapolated to about 0.25 mol of P_i/mol of S1 (Figure 6)].

Consider now the P_i burst experiments with actoS1 under single-turnover conditions (Figure 6a). There were two phases: a rapid rise followed by a slow one of kinetics near the overall k_{cat} of actoS1. This situation is most simply explained by there being a single type of cleavage step with actoS1 too. It seems unlikely that heterogeneity in the cleavage step of S1 explains the differences between the P_i transients of S1 and actoS1.

Another interpretation of our data is given by the scheme in Figure 7. This is based on three observations. First, the kinetics of the cleavage steps with S1 and actoS1 are different. Second, actoS1 ATPase follows a wholly dissociative pathway. Finally, the kinetics of the P_i release steps appear to be similar. We considered several schemes to take account of these observations before finally deciding on that given in Figure 7.

In Figure 7, we emphasize that the actin and ATP sites on S1 are separate [e.g., see Audemard et al. (1988) and Mornet et al. (1989)]: the actin site is represented by a circle and the ATPase site by a square or triangle. The lower pathway represents S1 ATPase.

The key feature of the scheme is that there is a transmission mechanism between the ATPase and actin binding sites as has already been proposed on structural grounds [see Botts et al. (1989) and references cited therein]. Thus, when actin binds to the actin site, there is a structural change to the ATPase site (shown by square to triangle). Conversely, when the

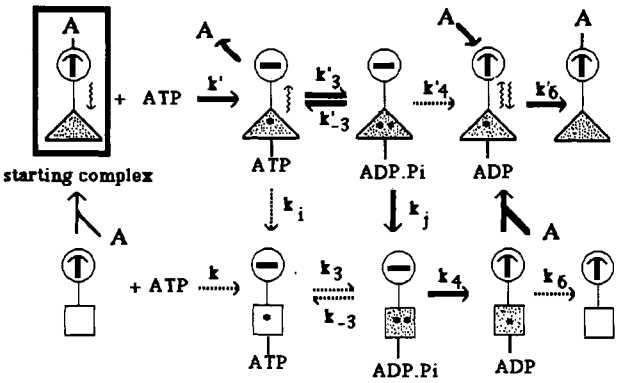

FIGURE 7: Pathway for actoS1 ATPase at −15 °C. The predominant pathway is shown by heavy arrows and dotted squares and triangles. For the sake of clarity, collision complexes are excluded. S1 is shown by a circle (actin site) joined to a square (ATP site in S1 alone) or a triangle (actin-induced ATP site). A circle with an horizontal bar indicates a "weak", or with an arrow, "strong", actin binding site. The asterisks denote different nucleotide-induced conformations of the ATP site. Wavy arrows show the direction of information flow between the sites. Kinetic constants pertaining to the triangular actin-induced conformation are shown by primes (e.g., k'_4).

ATPase site is occupied by ATP or ADP·P_i, the actin site changes from a "strong" (arrow in Figure 7) to a "weak" (horizontal bar) site.

Consider the initial steps of actoS1 ATPase. The starting material is actoS1 in which the ATPase site has the triangular actin-induced conformation. Upon the addition of ATP, the actin dissociates, but the triangular conformation remains: its return back to the square, actin-free conformation is relatively slow, i.e., $k_i < k'_3 + k'_{-3}$. Thus, at −15 °C, we propose that both the tight binding of ATP and the cleavage step take place with the actin-induced conformation of S1.

So far, ATP hydrolysis has proceeded in the absence of actin. However, there is a K_m for actin; i.e., the ATPase activity of the S1 increases hyperbolically with the actin concentration. Thus, actin returns to the pathway. Since the kinetics of the P_i release steps with S1 and actoS1 are similar, we propose that the triangle conformation in M·ADP·P_i changes rapidly to the square conformation, via k_j (i.e., $k_j \gg k'_4$). The P_i is now released via k_4, and it is at this stage that the actin returns. This return causes the S1 to change back to its triangle conformation. The kinetics of the release of ADP now occur via a rapid k'_6. With S1 alone at −15 °C, it is the ADP release kinetics (k_6) that are rate limiting: its increase by actin explains the increase in the k_{cat} of S1 (Table III). We were unable to obtain an estimate for k'_6, but it must be greater than 5×10^{-3} s^{-1} (k_{cat} for actoS1).

The scheme in Figure 7 was used to fit the data from the P_i burst experiments with actoS1 (Figure 6). The fits were good, and it is noteworthy that identical fits were obtained whether or not the S1 was saturated with actin. The constants obtained are summarized in Table III.

In conclusion, this is a continuation of a study on the physicochemical properties of the actin–S1–ATP system. As mentioned in the introduction, it is important to investigate the actoS1 system in this way. However, we are not yet in a position to extrapolate directly from our data, obtained from solution studies under cryoenzymic conditions, to the problem of the mechanism of muscle contraction.

The first aim of cryoenzymology is to slow down fast reactions, thus facilitating their study and often allowing for the accumulation of interesting intermediates. It was by this means that we obtained further kinetic information on the transmission mechanism of Morales [e.g., see Botts et al.

(1989)]. Further, we show that at −15 °C an important intermediate on actoS1 ATPase, M^{+}·ATP, accumulates with a half-life of several minutes. This stability could allow for its study by physical methods.

Cryoenzymology is also a means of perturbing a system. First, we show that the actin–S1 interaction remains strong in 40% ethylene glycol and at −15 °C. Second, the interaction of actin with M*·ATP appears to be weakened in 40% ethylene glycol. The ionic strength seems to modulate the effect of ethylene glycol: thus, at an ionic strength of 30 mM, Mushtaq and Greene (1989) found that ethylene glycol did not affect this interaction. Third, 40% ethylene glycol at −15 °C dramatically decreases the K_m for actin in S1 ATPase. This allows for studies of the actin–S1–ATP system at relatively low actin concentrations.

Acknowledgments

We thank J. L. Saldana for help in designing and building the necessary rapid reaction equipment.

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