# COMPLEX KINETICS OF ACTIN-SUBFRAGMENT-1 ATPase AT LOW TEMPERATURE

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## 1. Introduction

Actin and myosin are the two proteins directly involved in muscular contraction [1]. According to the current crossbridge hypothesis of contraction [2,3] muscle is a molecular machine in which ATP hydrolysis induces structural changes in actin–myosin complexes which are manifested macroscopically as contraction. Thus the study of the actin–myosin ATPase mechanism is fundamental to the understanding of muscular contraction.

The earliest measurements on the effect of actin on the ATPase activity of myosin, or its proteolytic derivative subfragment-1 (S-1), indicated that actin acted as a simple enzyme activator or modifier [4,5]. However later experiments, performed at temperatures close to 0°C, were not compatible with such a simple scheme: for instance the apparent turnover rate per actin was $\leqslant$ 10-times greater than the apparent turnover rate/S-1 [6,7]. These results were interpreted in terms of a 'refractory state' of myosin, unable to bind to actin [6]. However not all the data presented were compatible with this model and alternative explanations, such as the reversal of the dissociation reaction [8] were not considered.

In this study detailed measurements of the actin–S-1 ATPase activity at 25°C and 0°C under conditions of low ionic strength and physiological $H^+$ and $Mg^{2+}$ concentrations have been made. The object was to identify the ways in which the ATPase deviated from a model of simple enzyme activation and to interpret the observations in the light of what we know about the detailed mechanism of the actin–S-1 ATPase.

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## 2. Materials and methods

### 2.1. *Protein preparations*

Myosin and polymeric actin were prepared from rabbit back and leg muscle by standard methods [1,9–11]. Subfragment-1 was prepared by chymotryptic digestion of myosin [12] and purified by ammonium sulphate precipitation at 50–65% saturation. Before use both actin and S-1 were dialysed against the buffer used for ATPase assay and then clarified by a brief centrifugation at 40 000 × *g*. Molecular weights were assumed to be 110 000 for S-1 and 45 000 for actin momomer.

### 2.2. *ATPase measurements*

S-1 and actin were incubated in 0.5 ml 5 mM Tris, 5 mM morpholino-*N,N'*-ethane sulphonic acid (MES), pH 7.0, 1 mM $MgCl_2$, 0.1 mM $CaCl_2$, 0.3 mM cysteine. The reaction was started by adding MgATP to 2 mM and mixing vigorously for 1 s and was terminated by adding 0.5 ml 5% trichloroacetic acid. Incubation time was varied from 30–150 s so that not more than 20% ATP was hydrolysed. The phosphate liberated was assayed according to [13].

In order to measure the rate of ATP hydrolysis due to the actin–S-1 interaction when S-1 was in excess, the ATPase activity due to S-1 alone was measured under identical conditions and subtracted from the total ATPase activity. Since S-1 concentration was 5–120-times greater than actin concentration no correction was applied for S-1 bound to actin.

The subtraction was usually < 15% total except for the experiment with 1 μM actin ((●) in fig.2) where it reached 50% at the highest S-1 concentration used.

## 3. Results

### 3.1. *ATPase activity at 25°C*

Figure 1 shows that the ATPase activity/S-1 is activated by increasing actin concentrations and that the activation shows a good fit to the curve expected for simple saturating kinetics in agreement with [4,5]. It is also possible to fix the actin concentration and increase the S-1 concentration, in which case the ATPase activity due to the actin–S-1 interaction (i.e., after subtraction of the S-1 ATPase activity) shows simple saturating kinetics with respect to the S-1 concentration. The calculated apparent maximum velocities and Michaelis constants were quite similar for both curves (table 1).

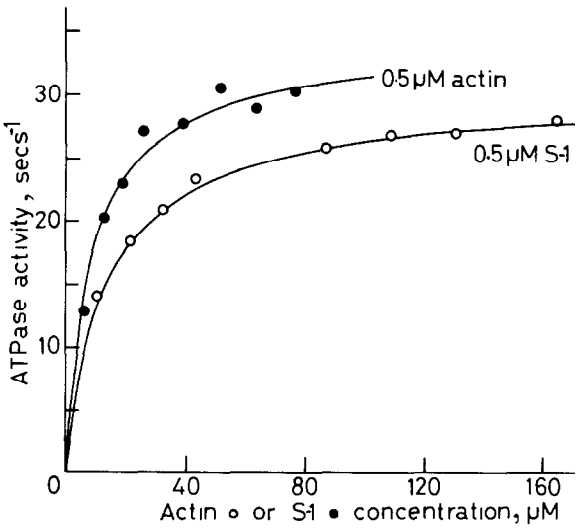


Fig.1. Actin–S-1 ATPase activity/S-1 at varying actin concentration (○) and / actin at varying S-1 concentration (●) at 25°C. Solid lines are the best fit of the data to the Michaelis-Menten equation [17]. Single pair of experiments using the same protein preparations.

### 3.2. *ATPase activity at 0°C with S-1 concentration limiting*

At low temperature the kinetics became complex. ATPase activity/S-1, measured at a constant 4 μM (S-1) did not show simple saturating kinetics as the actin concentration was increased. Activity reached a maximum at 50 μM actin and then decreased. Although the decrease was relatively small it was quite reproducible: this is shown by plotting the combined data from two experiments using two separate protein preparations in fig.2. It is unlikely that the decrease is an artifact of the assay procedure since identical experiments at 25°C did not give a biphasic curve.

### 3.3. *ATPase activity at 0°C with actin concentration limiting*

The ATPase activity per actin at 1 μM, 4 μM and 8 μM actin concentration showed simple saturating

Table 1
Estimated kinetic parameters of actin–S-1 ATPase

| Fixed species | | Variable species | Temp. (°C) | app. $K_m$ (μM) | app. $V_m$ ($s^{-1}$) |
|---|---|---|---|---|---|
| S-1 | 0.5 μM | Actin | 25 | 12.9 ± 0.6 | 23.8 ± 0.3 |
| Actin | 0.5 μM | S-1 | 25 | 9.4 ± 1.3 | 27.5 ± 0.9 |
| S-1 | 4 μM | Actin | 0 | ⩽ 4 | 0.45 ± 0.04[a] |
| Actin | 1 μM | S-1 | 0 | 14.1 ± 1.1 | 3.0 ± 0.06 |
| Actin | 4 μM | S-1 | 0 | 25.2 ± 1.7 | 3.1 ± 0.08 |
| Actin | 8 μM | S-1 | 0 | 41.7 ± 1.5 | 3.2 ± 0.06 |

[a] Data 0–30 μM actin only used. $K_m$ is approximate since S-1 concentration exceeds app. $K_m$

$K_m$, $V_m$ and standard errors calculated according to [17] from data in fig.1,2.

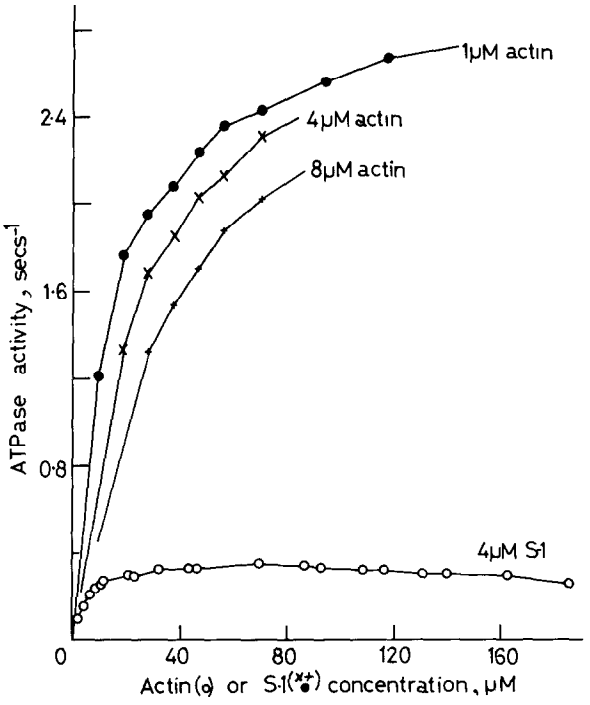


Fig.2. Actin–S-1 ATPase activity at 0°C. (○) rate/S-1 at 4 μM S-1 with varying actin concentration, (●) rate/actin for 1 μM actin with varying S-1 concentration. (X) As for 4 μM actin. (+) As for 8 μM actin.

kinetics as the S-1 concentration was increased (fig.2, table 1) but there were two anomalies:

1. In contrast to the 25°C result (fig.1), at 0°C the ATP turnover rate/actin ($V^s_m$) was 8-times greater than the turnover rate/S-1 ($V^a_m$). At 15°C the result was intermediate: $V^s_m$ was 5-times $V^a_m$.

2. The concentration dependence of activation by S-1 was different when measured with 1 μM, 4 μM and 8 μM fixed actin concentration (fig.2). Analysis of the curves showed clearly (table 1) that the app. $K_m$ ($K^{s'}_m$) increased with greater actin concentrations but the ATP turnover rate ($V^s_m$) remained constant. $V^s_m$ did not vary by more than ± 15% in 9 determinations. (3 protein preparations each measured at 3 actin concentrations). This result is at variance with [6] where both $K^{s'}_m$ and $V^s_m$ change under these conditions.

## 4. Discussion

Can the steady state actin–S-1 ATPase reaction be described as a simple enzyme activation process, as originally proposed [6]? If so, we should expect the Michaelis constant and ATP turnover rates obtained by titration of actin at constant S-1 concentration and of S-1 at constant actin concentration to be the same [4,6]. At 25°C this does occur (fig.1, table 1). Thus the reaction at 25°C can be adequately described by a simplification of the mechanism established by transient kinetic methods [3,8,14–16] thus:

$$\overset{**}{\text{S-1}}.\text{ADP}.\text{P}_i + \text{actin} \overset{c}{\rightleftharpoons} \text{actin}.\overset{**}{\text{S-1}}.\text{ADP}.\text{P}_i \xrightarrow{d} \text{products} + \text{S-1–actin} \quad (1)$$

in which formation of $\overset{**}{\text{S-1}}.\text{ADP}.\text{P}_i$ is presumed to be sufficiently fast and irreversible to have no observable effect on the steady state kinetics [3].

At 0°C this is clearly not the case: the turnover rate/S-1 is 12.5% turnover rate/actin. This suggests that only 12.5% S-1 molecules not bound to actin were in the state $\overset{**}{\text{S-1}}.\text{ADP}.\text{P}_i$ which is in direct equilibrium with actin [16]. This observation was explained [6] by proposing that the hydrolysis reaction, which forms $\overset{**}{\text{S-1}}.\text{ADP}.\text{P}_i$ from $\overset{*}{\text{S-1}}.\text{ATP}$ (step b in eq. (2)) is slow relative to step d. Recent direct measurement of the hydrolysis reaction at low temperature and ionic strength ([14,15], Geeves and Trentham, personal communication) have supported this hypothesis. The kinetic scheme should therefore be expanded to include the hydrolysis step which, if slow, will influence the steady state kinetics, thus:

$$\overset{*}{\text{S-1}}.\text{ATP} + \text{actin} \overset{b}{\rightleftharpoons} \overset{**}{\text{S-1}}.\text{ADP}.\text{P}_i + \text{actin} \overset{c}{\rightleftharpoons} \text{actin}.\overset{**}{\text{S-1}}.\text{ADP}.\text{P}_i \xrightarrow{d} \text{products} + \text{actin}.\text{S-1} \quad (2$$

This hypothesis will certainly explain the observed difference between $V^s_m$ and $V^a_m$ provided that the rate of hydrolysis (step b) is about 20% rate of step d, which is roughly comparable to the directly measured rates [14–16].

Can this hypothesis explain the other kinetic anomalies seen at low temperature? A steady state treatment of eq. (2) yields eq. (3) and eq. (4).

With S-1 concentration limiting:

$$\text{Rate/S-1} = \frac{V^a_m \cdot (\text{actin})}{K^a_m + (\text{actin})} \quad (3)$$

With actin concentration limiting:

$$\text{Rate/actin} = \left| \frac{V^s_m \cdot (\text{S-1})}{K^s_m\,(1 + (\text{actin})/K^s + (\text{S-1})} \right| \quad (4)$$

also it is found that $K^a_m = K^s_i$.

Equation (4) describes the experimental result obtained when actin concentration is limiting. The app. $K_m$ increased with increasing actin concentration but $V_m$ did not (table 2). If the data in fig.2 are applied to eq. (4) it may be calculated that $K^s_m = 10\ \mu M$ and $K^s_i = 2\ \mu M$ (which is about equal to est. $K^a_m \lesssim 4\ \mu M$).

Although a slow hydrolysis step can explain the experimental results, an alternative explanation cannot be ruled out. It should be noted that a simultaneous change in both $K^{s'}_m$ and $V^s_m$ when the limiting actin concentration is changed, which was reported in [6], but which was not found here, is not compatible with this hypothesis.

The proposed reaction scheme (eq. (2)) does not explain the biphasic curve obtained for actin activating the ATPase at a fixed S-1 concentration (fig.2) since the appropriate steady state equation (eq. (3)) predicts a simple hyperbola. Consideration of the known sequence of the actomyosin ATPase mechanism [3,14] shows that the only reasonable explanation is that there is significant reversal of the dissociation reaction:

$$\text{actin.S-1.ATP} \overset{a}{\rightleftharpoons} \text{actin} + \overset{*}{\text{S-1}}\text{.ATP}$$

at high actin concentrations [8]. When this additional step is included in the steady state analysis eq. (3) becomes:

$$\text{Rate/S-1} = \frac{V^a_m \cdot (\text{actin})}{K^a_m + (\text{actin}) + (\text{actin})^2/K^a_i} \quad (5)$$

This equation predicts a biphasic dependence of rate on actin concentration. Using the experimental data in fig.2 it may be calculated that $K^a_i$ is about 200 $\mu$M. An approximate value of the dissociation constant of the dissociation reaction, $K_a$, may be calculated from $K^a_i$ since it is roguhly equal to $K_a\,(1 + k_{+b}/k_{+d})$. If $k_{+b}/k_{+d}$ is 0.2 (see above) $K_a = 166\ \mu M$. Sleep and Hutton (personal communication) have obtained a similar value using an isotope exchange technique. If $K_{+a}$ is 1500 $s^{-1}$ [11], $k_{-a}$ is about $10^7\ M^{-1}\ s^{-1}$ which is very similar to the rate of the analogous reaction:

$$\text{actin} + \text{S-1} \longrightarrow \text{actin.S-1} \qquad [11,16]$$

In summary, therefore, in order to account for the steady state data at 0°C it is necessary to propose that the states actin.S-1.ATP, $\overset{*}{\text{S-1}}$.ATP, $\overset{**}{\text{S-1}}$.ADP.$P_i$ and actin.$\overset{**}{\text{S-1}}$.ADP.$P_i$ can all be significantly populated, whilst at 25°C the data require that only the states $\overset{**}{\text{S-1}}$.ADP.$P_i$ and actin.$\overset{**}{\text{S-1}}$.ADP.$P_i$ are populated. Presumably this occurs because there is a large relative increase in the rate of the hydrolysis step with increasing temperature.

Conditions in contracting muscle – high temperature and moderate ionic strength – would appear to favour $\overset{**}{\text{S-1}}$.ADP.$P_i$ and actin.$\overset{**}{\text{S-1}}$.ADP.$P_i$, being the only populated states, but the possibility of a 'refractory state' or reversal of dissociation in vivo should be considered when biochemical and mechanical data on whole muscle are interpreted [18,19].

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