The limiting rate of the ATP-mediated dissociation of actin from rabbit skeletal muscle myosin subfragment 1

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The ATP-induced dissociation of actoS1 has been studied at temperatures between −10°C and +30°C in a stopped-flow apparatus using ethylene glycol as antifreeze. At temperatures at and below 0°C the observed rate of the dissociation of actin shows a hyperbolic dependence on ATP concentration. This is interpreted in terms of a rapid binding of ATP followed by an isomerisation of the ternary complex which results in actin dissociation. Ethylene glycol weakens ATP binding but the rate of the isomerisation is unaffected. The second order rate constant for the dissociation shows a break in the Arrhenius plot.

Actomyosin dissociation *Arrhenius plot, discontinuous* *Subzero temperature stopped-flow* *Ethylene glycol*

1. INTRODUCTION

The sliding filament/cycling crossbridge model of muscle contraction has been developed from detailed structural, mechanical, and biochemical studies of muscle systems [1]. Any critical assessment of these models requires a detailed understanding of the dynamics of the interaction of myosin with actin as it progresses through each step of the hydrolytic cycle. The pathway of ATP hydrolysis by both myosin S1 and actoS1 from rabbit skeletal muscle has been defined using rapid flow and oxygen exchange methods [2,3]. However, the binding of ATP to actoS1 and the rate of the subsequently induced actin dissociation have not yielded to this approach, as the rate of the reactions involved lie beyond the range of conventional rapid-flow techniques [4].

We report here that the use of a solvent of 40% ethylene glycol has no significant effect upon the interaction of ATP with actoS1. The use of this solvent allows the ATP-induced actin dissociation reaction to be followed at <0°C where events involved can be directly observed in thermostated stopped-flow equipment. Earlier studies have measured the reaction directly using slow muscle myosin [5], and by estimating the rates of reactions $>500\ s^{-1}$ by measuring the loss in amplitude of the observed process [6,7]. This method relies on accurate measurement of instrument dead time and reaction amplitudes. We report here the first directly observed measurement of the maximum rate of the ATP-induced dissociation of actoS1 in rabbit skeletal muscle.

2. MATERIALS AND METHODS

F actin was prepared from an acetone powder as in [8] and concentrations were determined from $E^{1\%}_{280} = 11.08\ cm^{-1}$ and $M_r = 42000$ [9]. Myosin subfragment 1 was prepared from rabbit skeletal muscle as in [10] and concentrations are quoted on the basis of $M_r = 115000$ and $E^{1\%}_{280} = 7.9\ cm^{-1}$. Unless stated otherwise the experimental medium contained 4 μM actin, 5 μM S1, 0.1 M KCl, 5 mM free $MgCl_2$, 40% ethylene glycol and 50 or 200 mM imidazole or cacodylate buffer (pH 7.0). Cacodylate buffer was substituted for imidazole for experiments involving a large temperature range because of its solubility at low temperature and the very low temperature dependence of its protonic activity [11]. A high concentration of buf-

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fer was used to compensate for the very high concentrations of ATP required in the saturation experiments.

The first experiments were performed in a conventional stopped-flow spectrophotometer built in this laboratory and modified to allow for temperature control. The machine was built in an aluminium block through which coolant was circulated from an external thermostated bath, and the temperature of the block was measured with a Comark digital thermometer. The later experiments were performed on a stopped-flow spectrophotometer provided by Hi-Tech Scientific, in which temperature control to within 0.1°C was provided by using liquid nitrogen and a solid state thermostat. The rate of actoS1 dissociation was measured by following the change in 90° light scattering at 365 nm using a high pressure mercury lamp and a Farrand monochromator. Kinetic data were stored on a Data Lab DL 905 transient recorder and analysed on an ITT 2020 computer using non-linear least squares fitting routines as in [12].

3. RESULTS

The change in light scattering observed on mixing actoS1 with ATP at 25°C is shown in fig.1a fitted to a single exponential. At this temperature the observed rates of dissociation increase linearly with ATP concentration giving a second order rate constant of $1.5 \times 10^6\ M^{-1}.s^{-1}$ as shown in fig.1b. The second order rate constant shows a small linear decrease as ethylene glycol increases, dropping by a factor of two as the amount of ethylene glycol increases from 0–40%.

The effect of temperature on the second order rate constants with different buffer compositions is shown in fig.2, and the thermodynamic data calculated from this Arrhenius plot are shown in table 1. Under all conditions the activation energies at >5°C remain relatively constant varying from 35–55 $kJ.mol^{-1}$, indicating that the reaction mechanism is not altered either by the presence of 40% ethylene glycol or by varying the ionic strength. However, there is a sharp increase in the activation energy at low temperatures with a break point at 0–5 °C: the activation energy changing by a factor of 3 between the two temperature ranges.

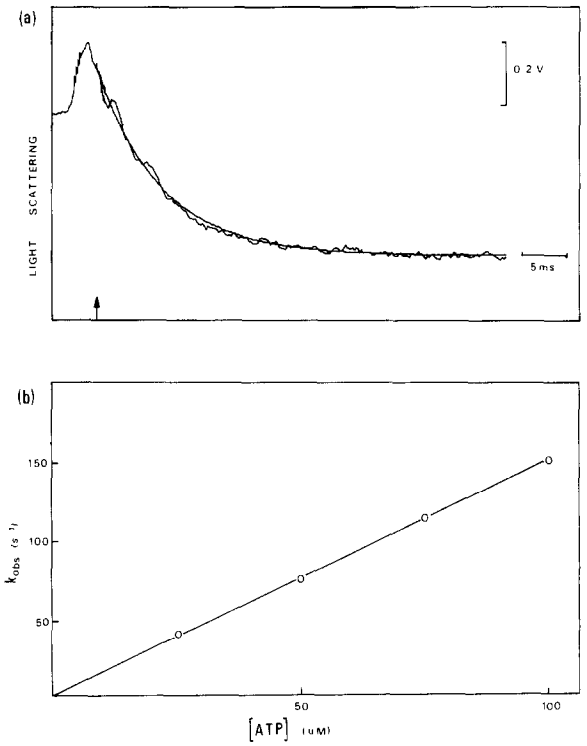

Fig.1. (a) Stopped-flow record of ATP-induced dissociation of actoS1 at 25°C: The trace is an average of 5 successive reactions and the superimposed exponential fit gives $k_{obs} = 74.7\ s^{-1}$. The arrow indicates the time at which flow stops. Conditions: 2 μM actoS1, 50 μM Mg^{2+}-ATP, 50 mM cacodylate (pH 7.0), 0.1 M KCl, 5 mM $MgCl_2$, 40% ethylene glycol (all reaction chamber concentrations). (b) Kinetic analysis of dissociation at 25°C: Linear fit gives the second order rate constant $K_1 \cdot k_{+2} = 1.49 \times 10^6\ M^{-1}.s^{-1}$. Conditions as in fig.1a.

At subzero temperatures in 40% ethylene glycol, the dependence of the observed rates on the concentration of ATP deviates from linearity. Typical results are shown in fig.3 and 4. The data in fig.4 give a good fit to a hyperbola which is the expected behaviour for a two-step dissociation reaction:

$$AM + ATP \underset{}{\overset{k_{+1}}{\rightleftharpoons}} AM \cdot ATP \overset{k_{+2}}{\rightleftharpoons} A + M \cdot ATP \qquad (1)$$

When ATP is in rapid equilibrium with the ternary complex and [ATP] ≫ [AM] then:

$$k_{(obs)} = \frac{K_1 . k_{+2} . [ATP]}{1 + K_1 . [ATP]} \qquad (I)$$

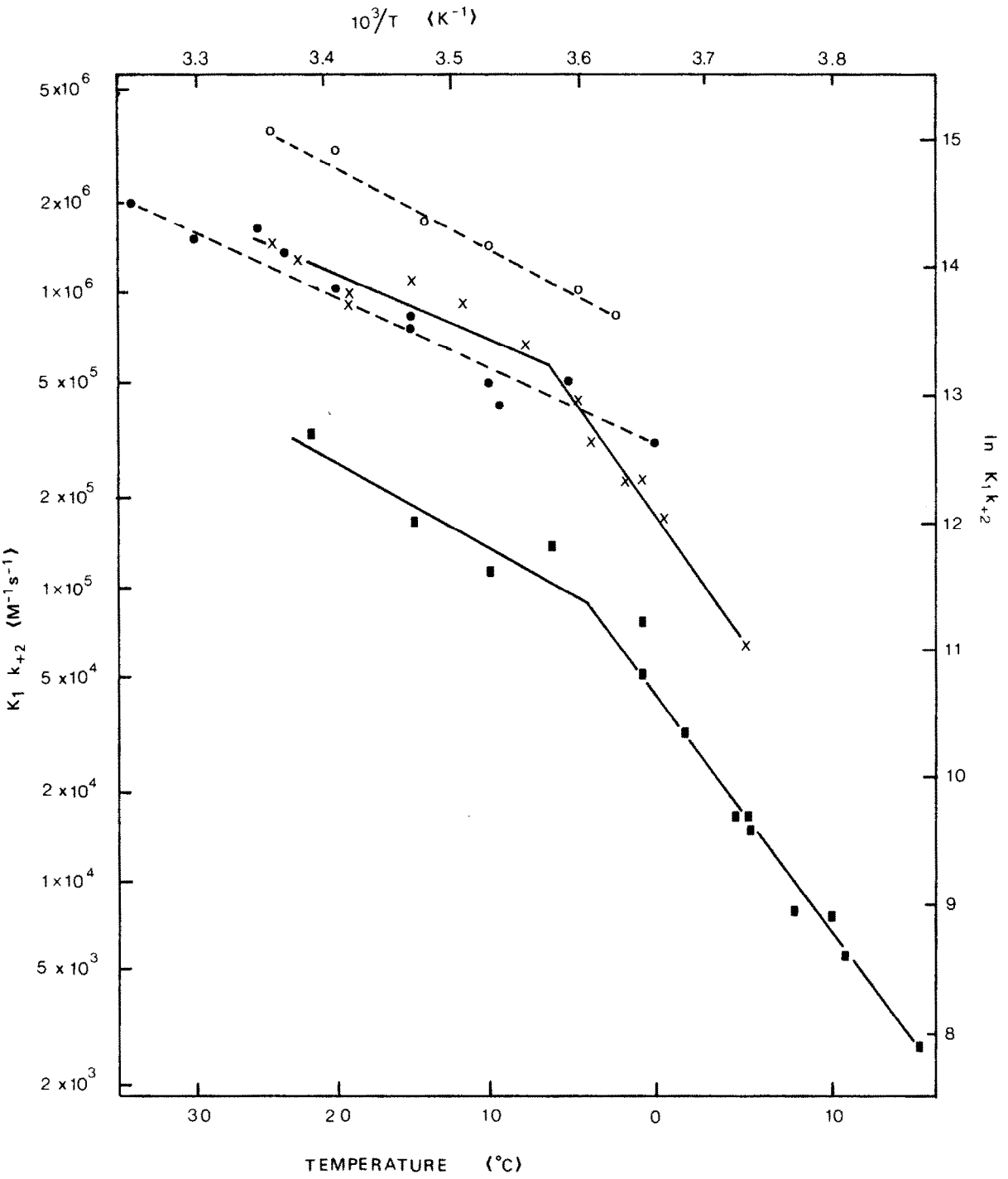

Fig.2. Arrhenius plot of the variation of the second order rate constant with temperature. Conditions: 0.1 M KCl, 5 mM $MgCl_2$ (pH 7.0), with: (○---○) 50 mM imidazole; (●---●) 50 mM imidazole, 40% ethylene glycol; (×---×) 50 mM cacodylate, 40% ethylene glycol; (■---■) 0.2 M cacodylate, 40% ethylene glycol.

Table 1

Thermodynamic data for the ATP-induced dissociation of actoS1

Conditions	Constant	E_A (kJ.mol^{-1})	$\Delta H^\dagger$ (kJ.mol^{-1})	$\Delta S^\dagger$ (J.mol^{-1}.K^{-1})	$\Delta G^\dagger$ (kJ.mol^{-1})
50 mM Imidazole, aqueous	$K_1 \cdot k_{+2}$	54.4	51.9	54.8	35.6
50 mM Imidazole, 40% ethylene glycol	$K_1 \cdot k_{+2}$	40.7	38.2	53.1	22.4
50 mM Cacodylate, 40% ethylene glycol	$K_1 \cdot k_{+2}$ >5°C	34.6	32.1	−19.1	37.8
50 mM Cacodylate, 40% ethylene glycol	$K_1 \cdot k_{+2}$ <5°C	116.7	114.2	273.0	32.9
200 mM Cacodylate, 40% ethylene glycol	$K_1 \cdot k_{+2}$ >5°C	45.9	43.4	7.2	41.3
	$K_1 \cdot k_{+2}$ <5°C	111.3	108.8	243.3	36.3
	k_{+2} <0°C	110.0	107.5	199.8	48.0

Conditions as for fig.2

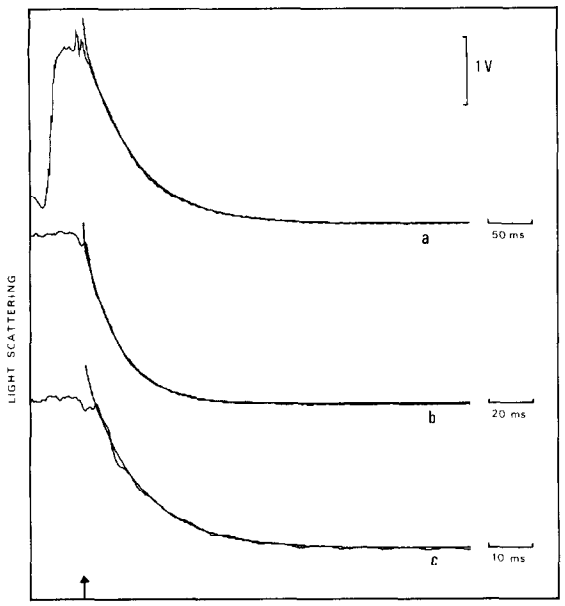

Fig.3. Stopped-flow records of the ATP-induced dissociation of actoS1 at −5°C. Each trace is the average of 5 successive reactions with an exponential fit superimposed. The arrow indicates the time at which flow stops. Conditions: 4 μM actoS1, 0.2 M cacodylate (pH 7.0), 0.1 M KCl, 5 mM $MgCl_2$, 40% ethylene glycol with: (a) 1 mM Mg^{2+}-ATP, k_{obs} = 18.3 s^{-1}; (b) 10 mM Mg^{2+}-ATP, k_{obs} = 65.8 s^{-1}; (c) 20 mM Mg^{2+}-ATP, k_{obs} = 85.6 s^{-1}.

The equilibrium constant for ATP-binding (K_1) is relatively insensitive to temperature, being about 100 ± 30 M^{-1} over the range −10°C to 0°C. The dissociation rate k_{+2} has a marked temperature dependence as shown in fig.5. The activation energy for this step (110 kJ.mol^{-1}) is the same as that for the observed second order rate constant for the whole process over this temperature range.

It is not possible to extend this plot much above 0°C as k_{+2} becomes too fast to be measured accurately, but at 0.5°C saturation can be observed in the absence of ethylene glycol as shown in fig.6. The values of K_1 and k_{+2} obtained (770 M^{-1} and 500 s^{-1}, respectively) indicate that the effect of ethylene glycol is predominantly on K_1 while k_{+2} is not greatly affected. Increasing the salt concentration in the absence of ethylene glycol at a 0.5°C shows that ionic strength has a similar effect, altering K_1 to 480 M^{-1} and k_{+2} to 435 s^{-1} in 0.2 M KCl.

4. DISCUSSION

There are two notable features of the results presented here:

(i) There is the sharp change in the slope of the Arrhenius plot of the second order rate constant;

(ii) The rate of the ATP-induced dissociation of

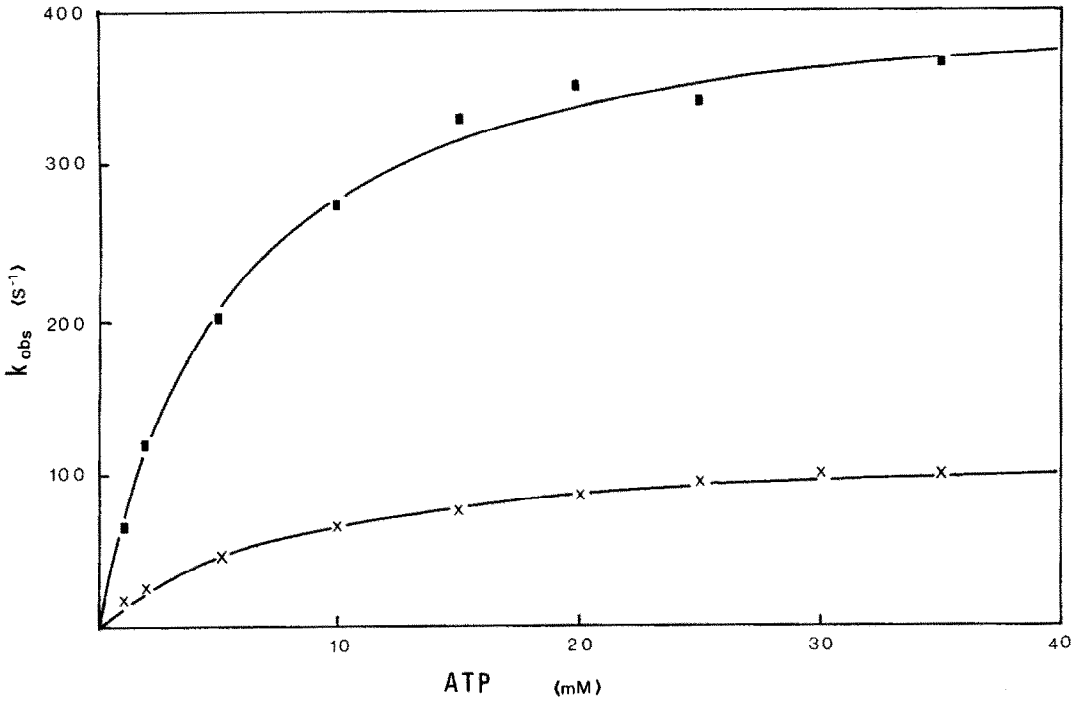

Fig.4. Kinetic analysis of dissociation at subzero temperatures. The data are fitted to hyperbolae by a least squares procedure using eq.(I). Conditions as in fig.3 with: (■) 0°C, $K_1 = 190\ M^{-1}$, $k_{+2} = 428\ s^{-1}$; (×) −5°C, $K_1 = 127\ M^{-1}$, $k_{+2} = 121\ s^{-1}$.

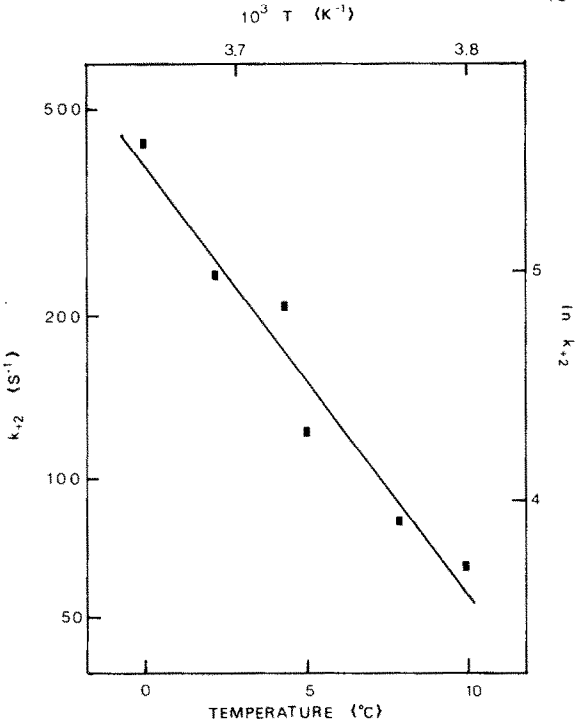

Fig.5. Arrhenius plot of the variation of k_{+2} with temperature; conditions as in fig.3.

actoS1 remains very fast in ethylene glycol which contrasts with the situation for S1 alone where the rate of ATP-binding is markedly reduced. At 15°C in low salt the rate of ATP-binding to S1 is reduced from about 160 s^{-1} in aqueous solution [13] to 16 s^{-1} in 40% ethylene glycol [14], whereas the rate of ATP-induced dissociation of actoS1 remains $>1000\ s^{-1}$ in both solvents.

A change in slope of an Arrhenius plot can be caused either by a change in the rate-limiting step of a process, or by a phase change in the system affecting the protein, the substrate or the solvent. A change in rate-limiting step is unlikely as this would normally give rise to a curved plot [15] rather than the sharp break observed here. Table 2 shows that such breaks in these plots are a common feature of studies with the myosin ATPase. It is remarkable that despite the wide range of studies listed, the break always occurs in the range 0–12°C and the activation energies either side of the break are very similar. This suggests that some common structural transition is being observed. From the

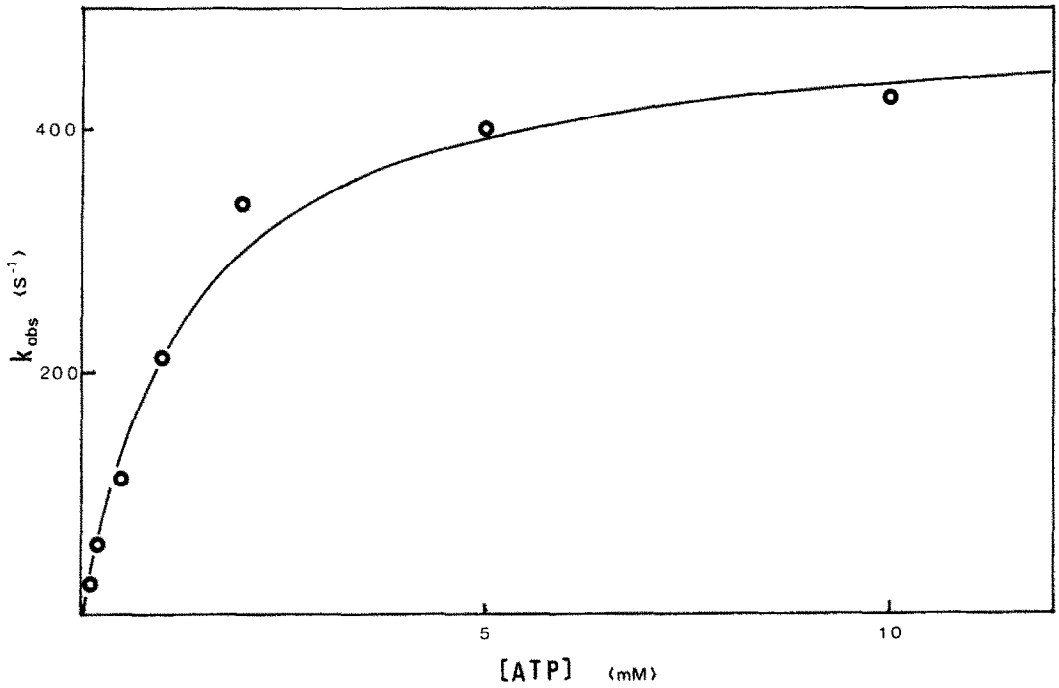

Fig.6. Kinetic analysis of dissociation in aqueous solution at 0.5°C. The data are fitted to a hyperbola by a least squares procedure using eq.(I). Conditions: 4 μM actoS1, 0.2 M cacodylate (pH 7.0), 0.1 M KCl, 5 mM $MgCl_2$; $K_1 = 770\ M^{-1}$, $k_{+2} = 500\ s^{-1}$.

Table 2

Comparison of data from Arrhenius plots of various myosin reactions

Reaction step	pH	[KCl] (mM)	Ethylene glycol	Break temp. (°C)	E_A above break ($kJ.mol^{-1}$)	E_A below break ($kJ.mol^{-1}$)	Ref.
Rate of actoS1 dissociation by ATP	7.0	100	40%	5	35	117	[Here]
Maximum rate of ATP-binding to S1	8.0	5	40%	12.5	40	120	[16]
k_{cat} for S1 Ca^{2+}-ATPase	7.5	250	40%	10	37	125	[17]
k_{cat} for S1 Mg^{2+}-ATPase	7.5	5	40%	5	60	121	[17]
k_{cat} for actoS1 Mg^{2+}-ATPase	7.5	5	40%	0	80	129	[17]
k_{cat} for HMM Mg^{2+}-ATPase	7.1	100	30%	0	65	120	[18]
k_{cat} for myosin ITPase	7.6	800	Aqueous solvent	11	36	170	[19]

thermodynamic data in table 1 it can be seen that $\Delta G^{\dagger}$ for the reaction remains relatively constant under all conditions, and the change in activation energy is compensated for by a large change in $\Delta S^{\dagger}$ above and below the break point indicating some change in order of the transition state of step 2 at this temperature.

From the experiments performed at 0.5°C it appears that k_{+2} is not affected by changes in ionic strength or ethylene glycol while K_1 does change, decreasing by a factor of 5 in the presence of 40% ethylene glycol and by a factor of 2 in 0.2 M KCl. Thus it would appear that the effect of solvent is principally on K_1 while temperature mainly affects k_{+2}. Since K_1 is insensitive to temperature and the temperature dependence of the second order rate constant is dominated by k_{+2}, the simplest assumption is that k_{+2} has a similar temperature dependence to the second order rate constant and has a break point at 5°C with a lower activation energy at room temperature. This would give a value of 500 M^{-1} for K_1 and 5000 s^{-1} for k_{+2} at 20°C using the value for the second order rate constant of 2.5×10^6 $M^{-1}.s^{-1}$. If the temperature dependence of k_{+2} remains constant, then it would have a value of 18000 s^{-1} at 20°C and K_1 would be 140 M^{-1}. These figures compare with values of K_1 = 1000–2000 M^{-1} and k_{+2} = 1500–2000 s^{-1} for cardiac S1 [7], K_1 = 333 M^{-1} and k_{+2} = 120 s^{-1} for arterial myosin (0.5 M KCl) [6], and K_1 = 4000 M^{-1} and k_{+2} = 1500 s^{-1} for chicken slow red, cardiac and smooth muscle S1 [8].

This assumes that the mechanism is that shown in eq.(1). There are several reasons for believing the mechanism to be more complex. Eccleston et al. [6] in their work on the dissociation of arterial actomyosin, discussed two- and three-step models of dissociation and favoured the three-step model shown in:

$$\mathrm{AM} + \mathrm{ATP} \overset{k'_{+1}}{\rightleftharpoons} \mathrm{AM}\cdot\mathrm{ATP} \overset{k'_{+2}}{\rightleftharpoons}$$

$$\mathrm{AM}'\cdot\mathrm{ATP} \overset{k'_{+3}}{\rightleftharpoons} \mathrm{A} + \mathrm{M}'\cdot\mathrm{ATP} \qquad (2)$$

In this case K'_1 is the rapid equilibrium for the formation of the ternary complex and k'_{+2} is the rate of the isomerisation of this complex; k'_{+3}, the rate of actin dissociation, would have to be greater than k'_{+2} at 0.5°C (i.e., >500 s^{-1}), and >1000 s^{-1} at room temperature. Assuming the reverse reaction is negligible, then:

$$k_{(\mathrm{obs})} = \frac{K'_1 \cdot k'_{+2} \cdot [\mathrm{ATP}]}{1 + K'_1 \cdot [\mathrm{ATP}]} \qquad (\mathrm{II})$$

Our data support this mechanism in two ways:

(i) The high temperature dependence of the maximum observed rate (k_{+2} or k'_{+2}) suggests a protein conformation change rather than a dissociation step;

(ii) Consideration of the binding of actin and ATP to S1 sets limits on the values of the binding constants:

$$\begin{array}{ccc} \mathrm{M} & \overset{b}{\longleftrightarrow} & \mathrm{AM} \\ {\scriptstyle a}\uparrow\downarrow & & \uparrow\downarrow{\scriptstyle c} \\ \mathrm{M}\cdot\mathrm{ATP} & \underset{d}{\longleftrightarrow} & \mathrm{AM}\cdot\mathrm{ATP} \end{array} \qquad (3)$$

In (3) $K_a = 10^{11}$ M^{-1} [3], and $K_b = 10^7$ M^{-1} [20]. The data here give a value of 10^3 M^{-1} for K_c, defining K_d as 10^{-1} M^{-1}. This is an unacceptably small value, as at low ionic strength this binding constant has been measured as 2×10^4 M^{-1} [21]. This discrepancy is avoided if an isomerisation of the ternary complex is allowed as defined by (2), where $K_c = K'_1 K'_2$. Limits can be set on the value of the equilibrium constant of the isomerisation step K'_2. At the ionic strength used here ATP causes complete dissociation of actoS1 at protein concentrations up to 10^{-4} M. Therefore $K_d < 10^4$, M^{-1} this then defines $K_c < 10^8$ M^{-1}, and as K'_1 is defined by our data as 10^3 M^{-1}, then $K'_2 < 10^5$.

This analysis assumes that the M·ATP state produced on actin dissociation is the same as that formed on binding ATP to S1. In this respect it is of interest to consider the effect of ethylene glycol on the rate of ATP-binding to both S1 and actoS1. The results presented here show that ethylene glycol has only a small effect on the observed rate of ATP-induced dissociation of actoS1. This is in marked contrast to the results in [14] where it was found that in low salt (5 mM KCl), ATP-binding to S1 alone is markedly affected by ethylene glycol. Basing their analysis on the two-step binding

model in [22], the authors found that K_1 (the rapid equilibrium of the initial S1–ATP complex) increases from 5×10^3 M^{-1} to 1.25×10^5 M^{-1} in the presence of 40% ethylene glycol, and k_{+2} (the rate of isomerisation of the binary complex) decreases from 160 s^{-1} to 16 s^{-1}. At higher ionic strength (150 mM KCl), and in 40% ethylene glycol, the maximum rate of formation of myosin-bound ATP is 45 s^{-1} when ATP binds to S1 alone and this compares with 5000 s^{-1} when ATP dissociates actoS1. Thus either the presence of actin causes a marked acceleration of the rate of formation of myosin-bound ATP or the myosin-bound ATP formed on release of actin is not the same as that formed on ATP binding to S1 alone. Measurement of the rate of ATP hydrolysis in the presence and absence of actin under conditions where the rate of ATP binding to S1 is rate-limiting for the hydrolysis step will distinguish the two possibilities.

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