Protein fluorescence changes associated with ATP and adenosine 5'-[γ -thio]triphosphate binding to skeletal muscle myosin subfragment 1 and actomyosin subfragment 1

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1. The fluorescence changes accompanying the binding of ATP and adenosine 5'-[γ -thio]triphosphate (ATP γ S) to myosin subfragment 1 (S1) and actomyosin subfragment 1 (actoS1) have been reinvestigated at 20 °C and 1 °C, pH 7.0, 0.1 M-KCl. 2. Two successive fluorescence enhancements are observed following ATP binding to both S1 and actoS1. 3. The slow fluorescence change has the same rate with S1 and actoS1, and is due to the ATP cleavage step. 4. With actoS1 the fast fluorescence change occurs after dissociation, so a new intermediate, S1 $\dagger$ ATP, is required on the actoS1 pathway. 5. The dissociation of actoS1 by ATP γ S results in a fluorescence enhancement with the same apparent rate as dissociation, but indirect evidence suggests that this too occurs on a dissociated state.

INTRODUCTION

Investigations of the myosin subfragment 1 (S1) and actoS1 ATPase mechanisms in solution, under conditions where complete dissociation takes place, have led to the following equation for the steps involving ATP binding and cleavage:

where asterisks denote states with enhanced fluorescence. The first nucleotide-bound intermediates (S1 $\cdot$ ATP and actoS1 $\cdot$ ATP) are collision complexes, and $K_1 = 4 \times 10^3 \text{ M}^{-1} \cdot \text{s}^{-1}$, $k_{+2} > 1000 \text{ s}^{-1}$, $k_{+3} + k_{-3} = 125 \text{ s}^{-1}$, $K_4 = 10^3 \text{ M}^{-1}$, $k_{+5} = 5000 \text{ s}^{-1}$ and $K_6 = 10^6 \text{ M}^{-1}$, at 20 °C, 0.1 M-KCl, pH 7–8 (Johnson & Taylor, 1978; Chock *et al.*, 1979; Geeves & Trentham, 1982; Millar & Geeves, 1983).

Two fluorescence enhancements accompany the binding of ATP to S1 (Johnson & Taylor, 1978; Chock *et al.*, 1979). The slower of the two has the same rate as the phosphate burst, and so has been assigned to the ATP cleavage step. Under conditions where comparisons are possible (generally low ionic strength, low temperature and low pH), the rate of the fast phase is approximately the same as the rate of tight binding of ATP to S1, measured by the cold chase technique. The amplitude of this phase is equal to that observed when ADP[NH]P, ATP γ S or ADP bind to S1. The fast fluorescence phase has therefore been assigned to the isomerization which accompanies the binding of ATP (step 2 of eqn. 1). The

rate of this step is difficult to measure at normal ionic strength and temperature, since at high ATP concentrations it is fast ($> 1000 \text{ s}^{-1}$ at 20 °C), and at low ATP concentrations it is difficult to resolve from the slower fluorescence change that accompanies cleavage.

Millar & Geeves (1983) investigated the ATP-mediated dissociation of actoS1 using light scattering, and found

that ATP binding to actoS1 was followed by a rate-limiting isomerization of the ternary (actin $\cdot$ S1 $\cdot$ ATP) complex (step 5 in eqn. 1). Geeves *et al.* (1986) showed that this isomerization was accompanied by a change in fluorescence of pyrene-labelled actin. Under conditions where complete dissociation takes place (protein concentrations $< 10 \mu\text{M}$ and 0.1 M ionic strength), the cleavage step is identical to that with S1 alone. This has been demonstrated many times (Johnson & Taylor, 1978; Stein *et al.*, 1981; Geeves & Trentham, 1982). It has therefore been assumed that the species formed when actin dissociates from the ternary complex is the same as that formed when ATP binds to S1 alone (i.e. S1 $\cdot$ ATP).

The fluorescence changes accompanying the binding of ATP to actoS1 have been studied by Johnson & Taylor (1978). They observed a single-phase fluorescence enhancement which correlated with the slow S1 fluorescence phase (and therefore the common cleavage step). They also measured an increase in fluorescence when actin bound to S1, forming a species actoS1 $\cdot$ with a similar fluorescence to S1 $\cdot$ ATP. They therefore proposed

Abbreviations used: S1, myosin subfragment 1; actoS1, actomyosin subfragment 1; ATP γ S, adenosine 5'-[γ -thio]triphosphate; ADP[NH]P, adenosine 5'-[β -imido]triphosphate; ϵ ATP, 1, N^ϵ -ethenoadenosine triphosphate.

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that no fast fluorescence phase was observed with the ATP-mediated dissociation of actoS1 because an increase in fluorescence due to the binding of ATP to actoS1 (equivalent to the fast phase with S1) was largely cancelled out by a simultaneous (and slightly smaller) decrease in fluorescence due to actoS1 dissociation. The actual fluorescence of S1*ATP is thus the same starting from S1 or actoS1. The binding of ATP to actoS1 has also been investigated by Biosca *et al.* (1984) using cold chase at 15 °C, 10 mM-KCl. They obtained a maximum rate for tight binding of 63 s^{-1} , which was slower than the rate of light scattering, and therefore occurred after actin dissociation. They concluded that the first dissociated species was different from S1*ATP, and that actoS1 dissociation was followed by an isomerization of the binary complex to form S1*ATP.

There are thus three possible steps where a fast fluorescence enhancement could occur. (1) It could occur at the dissociation step, as suggested by Johnson & Taylor (1978). (2) It could occur after dissociation but before cleavage. This would be compatible with the binding data of Biosca *et al.* (1984). (3) It could occur before dissociation. This is suggested by the studies of Trybus & Taylor (1982) on the ADP[NH]P-mediated dissociation of actoS1 in 0.6 M-KCl, where a fast fluorescence enhancement is seen prior to dissociation. In this work the fluorescence changes associated with ATP binding to S1 and actoS1 have been reinvestigated in an attempt to identify which of these alternatives are correct. The binding of ATP γ S to S1 and actoS1 is also investigated, since the fluorescence change due to binding is easily separated from the very slow cleavage step, and the rate of actoS1 dissociation is 10 times slower than with ATP (Goody & Hofmann, 1980; Geeves *et al.*, 1986). It is concluded that the actoS1 fast fluorescence change takes place after dissociation, and an additional S1·ATP intermediate is required on the actoS1 pathway.

MATERIALS AND METHODS

Proteins and nucleotides

Myosin subfragment 1 was prepared from rabbit skeletal muscle by the method of Weeds & Taylor (1975), and S1-A1 was separated from S1-A2 by ion-exchange chromatography on DEAE Sephadex. Purity of the isoenzyme was assayed by SDS/polyacrylamide-gel electrophoresis, and S1-A1 was used throughout this work. F-actin was prepared from an acetone powder of rabbit skeletal muscle as described by Lehrer & Kerwar (1972). Protein concentrations were calculated using M_r 115000, $\epsilon_{280}^{1\%}$ 7.9 cm^{-1} for S1 and M_r 42000, $\epsilon_{280}^{1\%}$ 11.08 cm^{-1} for actin. ATP and ATP γ S were obtained from BCL, and the ATP was used without further purification. ATP γ S was purified by ion-exchange chromatography on a DEAE-cellulose column eluted with a linear gradient of 0–0.6 M-triethylammonium bicarbonate buffer, pH 7.6. The purity of the ATP γ S was assayed qualitatively by t.l.c. on PEI MN-polygram cellulose 300 UV plates (Camlab, Cambridge, U.K.) using a solvent of 0.75 M-KH₂PO₄/HCl, pH 3.4 (Goody & Eckstein, 1971). The concentration of the nucleotides was determined using the molar absorption coefficient at 259 nm of $15.4 \times 10^3\text{ M}^{-1}\cdot\text{cm}^{-1}$. All the experiments described in this paper were performed in a buffer consisting of 50 mM-cacodylate (pH 7.0)/0.1 M-KCl/

5 mM-MgCl₂. Concentrations throughout are quoted as reaction chamber concentrations.

Apparatus

The rapid mixing experiments were carried out on a Hi-Tech Scientific SF3L stopped flow spectrophotometer which was thermostatically controlled to within 0.1 °C. The optical system consisted of a mercury lamp with a Bausch and Lomb monochromator, and 90° emitted light was detected by an EMI 9526B photomultiplier. Light scattering measurements were made at 365 nm. For tryptophan fluorescence measurements the exciting wavelength was changed to 294 nm, a liquid optical filter consisting of a 10 mm path of saturated NiSO₄/CoSO₄ solution was introduced into the exciting beam, and a second filter consisting of a 5 mm path of acetone (BDH Spectrosol grade), together with a Schott glass filter type UG11, was introduced in front of the photomultiplier to cut out scattered exciting wavelengths. The signal from the photomultiplier was digitized as 1024 8-bit data points by a Datalab DL905 transient recorder and then analysed on an ITT 2020 microcomputer using standard non-linear fitting programs.

RESULTS

S1 and ATP γ S

The stopped flow fluorescence records of ATP γ S binding to S1 at 20 °C and 1 °C are shown in Figs. 1(b) and 1(d) fitted to single exponentials, and the concentration dependence of the observed rates and amplitudes are shown in Figs. 2 and 3. The rate of the ATP γ S cleavage step has been measured as 0.24 s^{-1} at 21 °C, 0.1 M-KCl, pH 8 (Bagshaw *et al.*, 1972), and 0.014 s^{-1} at 4 °C, 10 mM-KCl, pH 8 (Goody & Mannherz, 1975), so the cleavage step is far too slow to be contributing to these fluorescence signals. As expected, the rate of the fluorescence enhancement shows a hyperbolic dependence on ATP γ S concentration at both 20 °C and 1 °C, and the amplitude does not vary with ATP γ S concentration. This is consistent with a two-step binding process, where only the second step is contributing to the observed fluorescence change.

We were unable to use a sufficiently high concentration of ATP γ S to give an accurate measure of the maximum rate of ATP γ S binding at either temperature, but hyperbolic fits to the data suggest values of 340 s^{-1} at 20 °C and 20 s^{-1} at 1 °C. Bagshaw *et al.* (1974) obtained a maximum fluorescence rate of 300 s^{-1} at pH 8.0, 23 °C. These results are summarized in Table 1. At 1 °C the amplitude of the fluorescence change is about half that observed at 20 °C (4 % relative to S1 rather than 9 %). It will be seen that this reduction in amplitude at 1 °C is a common feature, and its implication will be discussed later.

S1 and ATP

The results of mixing ATP with S1 are shown in Figs. 1(a) and 1(c), and Figs. 2 and 3. The data are in good agreement with the findings of Johnson & Taylor (1978) and Chock *et al.* (1979). At 20 °C (Fig. 2), the decrease in both rates and amplitude of the fluorescence enhancement at high ATP concentrations indicates that although the observed fluorescence signal gives a reasonable fit to a single exponential, it is actually composed of two phases, each with an amplitude of about 10 %. The fast

Fig. 1. Stopped-flow fluorescence records of ATP and ATP γ S binding to S1

Conditions: 50 mM-cacodylate (pH 7.0)/0.1 M-KCl/5 mM-MgCl₂/5 μ M-S1, with (a) 1 mM-ATP, 20.0 °C, k_{obs} 71.5 s⁻¹, amplitude 11.5%; (b) 1 mM-ATP γ S, 20.0 °C, k_{obs} 280 s⁻¹, amplitude 8.6%; (c) 1 mM-ATP, 1.0 °C, k_{obs} 5.75 s⁻¹, amplitude 10.1%; (d) 1 mM-ATP γ S, 1.0 °C, k_{obs} 14.6 s⁻¹, amplitude 3.8%. Time zero is the point at which flow stops. Amplitudes are quoted relative to S1.

phase is complete within the dead time at high concentrations, but is observed indirectly at low ATP concentrations. The observed rate and amplitude at high concentrations are therefore due almost entirely to the slow phase. At 20 °C this rate is 90 s⁻¹, which compares with values of 125 s⁻¹ obtained by Johnson & Taylor (1978) under the same conditions, and 63 s⁻¹ at pH 6.4, 0.1 M-KCl, 20 °C (Chock *et al.*, 1979). The rate of the initial phosphate burst has been measured as 100 s⁻¹ at 20 °C, pH 8.0, 80 mM-KCl (Geeves & Trentham, 1982), and as 90 s⁻¹ at 15 °C, 150 mM-KCl, pH 8 (Barman *et al.*, 1983), so as expected there is reasonable correlation between the slow fluorescence transient and the cleavage step. The rate of the fast phase is too fast to measure, but from the loss of amplitude in the dead time (0.9 ms) it must be faster than 1000 s⁻¹. Johnson & Taylor (1978) also estimated the rate of the fast fluorescence phase to be at least 1500–2000 s⁻¹ at 20 °C.

The ATP-binding kinetics are different at 1 °C. The rates appear to show a hyperbolic dependence on ATP concentration, and the amplitude is constant, so there is apparently no indication of a fast phase. The rate at high ATP concentration (6 s⁻¹) is a reasonable value for the rate of the cleavage step at this temperature, since Sleep

& Taylor (1976) obtained a rate of 7 s⁻¹ for the phosphate burst at 3 °C, 40 mM-KCl, pH 6.9. Furthermore, the reduction in the amplitude of this (slow) fluorescence enhancement from 10% at 20 °C to 8% at 1 °C is consistent with the observation that the magnitude of the phosphate burst decreases with temperature (Taylor, 1977; Kodama *et al.*, 1986). Thus the observed fluorescence signal at 1 °C is due to the cleavage step. Johnson & Taylor (1978) observed a decrease in the observed fluorescence amplitude at high ATP concentrations at 5 °C as well as at 20 °C. They also observed a lag in the fluorescence transient at low ATP concentrations and a burst at high concentrations at 5 °C, which they took as further evidence for a fast phase. They were unable to observe this phase directly, but estimated that it had a rate of 300–400 s⁻¹. Lag and burst phases could be difficult to detect in our data, but a change in signal amplitude should have been observable, and no such change was seen. However, by analogy with ATP γ S, the amplitude of a fast phase would be only 4% at 1 °C, and therefore difficult to detect. If this decrease in fluorescence amplitude occurred between 5 °C and 1 °C, this could explain why a fast phase was observed by Johnson & Taylor (1978) but not by ourselves.

Fig. 2. Concentration-dependence of the fluorescence change for ATP and ATP γ S binding to S1 at 20 °C

Conditions as Fig. 1. $\circ$, ATP; $\bullet$, ATP γ S. The curve through the ATP rates is drawn by hand, and the curve through the ATP γ S rates is the best fit to eqn. (1) with K_1 4500 M $^{-1}$, k_{+2} 340 s $^{-1}$.

Fig. 3. Concentration-dependence of the fluorescence change for ATP and ATP γ S binding to S1 at 1 °C

Conditions as Fig. 1. $\circ$, ATP; $\bullet$, ATP γ S. The curve through the ATP rates is drawn by hand, and the curve through the ATP γ S rates is the best fit to eqn. (1) with K_1 1800 M $^{-1}$, k_{+2} 20 s $^{-1}$.

ActoS1 and ATP γ S

The fluorescence and light-scattering changes observed when ATP γ S dissociates actoS1 are shown in Figs. 4–6. All the fluorescence and light scattering transients give reasonable fits to single exponentials, and the rates of both light scattering and fluorescence changes have a hyperbolic dependence upon ATP γ S concentration. The rate of the fluorescence enhancement is the same, within experimental error, as the rate of actin dissociation. The values obtained for the apparent binding constant and maximum rate at 20 °C (1000 M $^{-1}$ and 380 s $^{-1}$ respectively) are very similar to those obtained by Goody & Hofmann (1980) using light scattering at 23 °C, 0.1 M-KCl, pH 8 (830 M $^{-1}$ and 380 s $^{-1}$ respectively). The amplitude of the fluorescence enhancement decreases with temperature from 9% at 20 °C to 4% at 1 °C in exactly the same way as with S1 alone, although the maximum rate with actoS1 is faster than that observed with S1. The amplitude of the light-scattering signal is constant, and the same as that observed with ATP (see below), indicating 100% dissociation.

Geeves *et al.* (1986) have shown that the rate of dissociation is controlled by the rate of the preceding isomerization step. Since the fluorescence signal has the

same observed rate as dissociation, the fluorescence change could therefore occur at the isomerization step, at the dissociation step, or at a subsequent fast step. Other evidence suggests that the ATP γ S fluorescence change probably occurs after dissociation, and the observed rate is limited by the rate of dissociation (or rather by the preceding isomerization). A preliminary experiment performed at –10 °C in the presence of 40% ethylene glycol showed that the maximum rate of the ATP γ S-induced dissociation of actoS1 was 18 s $^{-1}$, but the fluorescence enhancement occurred with a rate of only 2 s $^{-1}$. So at this temperature the two processes can be resolved, and the ATP γ S fluorescence change takes place after dissociation.

ActoS1 and ATP

The stopped flow fluorescence and light scattering records for the ATP-mediated dissociation of actoS1 at 20 °C (Fig. 4) give reasonable fits to single exponentials. The concentration dependence of the fluorescence rates and amplitudes (Fig. 5) is similar to that for S1 alone, in that both decrease at high ATP concentrations. As before this indicates that the signal is composed of two phases, each with an amplitude of 10% (relative to

Table 1. Summary of the kinetic constants for the binding of ATP and ATP γ S to S1 and actoS1 at 20 °C and 1 °C

The rates of the phosphate burst are obtained from quench-flow data under the most similar conditions available in the following references: ^aBagshaw *et al.* (1972), 20 °C, 0.1 M-KCl, pH 8; ^bGoody & Mannherz (1975), 4 °C, 10 mM-KCl, pH 8; ^cGeeves & Trentham (1982), 20 °C, 10 mM-KCl, pH 8; ^dSleep & Taylor (1976), 3 °C, 40 mM-KCl, pH 6.9.

Signal observed	Nucleotide	Temperature (°C)	Number of phases	Fast phase			Slow phase		
				Second-order rate constant (M ⁻¹ ·s ⁻¹)	Amplitude (%)	Apparent-binding constant (M ⁻¹)	Maximum rate (s ⁻¹)	Amplitude (%)	Rate of phosphate burst (s ⁻¹)
Fluorescence (S1)	ATP γ S	20	One	1.5×10^6	9	~ 4500	~ 340	None	0.24 ^a
		1	One	3.7×10^4	4	~ 1800	~ 20	None	$< 0.014^b$
	ATP	20	Evidence for two	1.0×10^6	10	< 1000	> 1000	10	100 ^c
Fluorescence (actoS1)		1	Evidence for two	?	4?	?	?	8	7 ^d
	ATP γ S	20	One	3.8×10^5	9	1000	380	None	
		1	One	2.1×10^5	4	1900	110	None	
Light scattering (actoS1)	ATP	20	Evidence for two	1.5×10^6	10	—	> 1000	10	130
		1	Two	3.0×10^5	4	—	120	7	6
	ATP γ S	20	One	3.8×10^5	—	1000	380		
ATP		1	One	2.1×10^5	—	1900	110		
		20	One	2.5×10^6	—	500	5000		
		1	One	5.6×10^5	—	800	700

Fig. 4. Stopped-flow fluorescence and light-scattering records of ATP and ATP γ S binding to actoS1

Conditions: 50 mM-cacodylate (pH 7.0)/0.1 M-KCl/5 mM-MgCl₂/5 μ M-S1/6 μ M-actin, with (a) 1 mM-ATP, 20.0 °C, k_{obs} 177 s⁻¹, amplitude 11.6%, calculated rate of light scattering at this concentration 1667 s⁻¹ (not shown); (b) 1 mM-ATP γ S, 20.0 °C, k_{obs} 204 s⁻¹, amplitude 12.4%, rate of light scattering 195 s⁻¹ (decreasing signal); (c) 1 mM-ATP, 1.0 °C, k_{obs} 5.57 s⁻¹, amplitude 5.4% for the slow phase, and k_{obs} 95.9 s⁻¹, amplitude 3.5% for the fast phase, rate of light scattering 329 s⁻¹ (not shown); (d) 1 mM-ATP γ S, 1.0 °C, k_{obs} 71.4 s⁻¹, amplitude 4.6%, rate of light scattering 69 s⁻¹ (decreasing signal). The fluorescence amplitudes are all relative to actoS1. The light-scattering amplitudes are scaled arbitrarily to match the fluorescence records. Time zero is the point at which flow stops.

actoS1). At 1 °C the light scattering records still fit single exponentials, but the fluorescence signal is now clearly seen to be biphasic (Figs. 4 and 6). The amplitudes of the two fluorescence phases are 4% for the fast phase and 7% for the slow phase, so they are both smaller than at 20 °C. The slow fluorescence phase at 1 °C has a similar rate and amplitude to the slow fluorescence change seen with S1 alone (6 s⁻¹, 7% with actoS1, 5.5 s⁻¹, 8% with S1), and neither the rate nor the amplitude vary with ATP concentration. It is therefore attributed to the common cleavage step. Once again the size of the slow fluorescence enhancement is reduced at 1 °C, reflecting the smaller size of the phosphate burst. The fast phase has a maximum rate of 120 s⁻¹, but there is no equivalent fast phase with S1 alone at 1 °C to compare this with.

At 20 °C, the observed fluorescence rate is slightly faster with actoS1 than with S1, but the rates converge at high ATP concentrations, and are reasonably similar at 2 mM-ATP (130 s⁻¹ with actoS1, 90 s⁻¹ with S1). Since the slow component of the observed fluorescence signal (due to the cleavage step) is the same in both cases, the difference must be due to a difference in the fast

component with S1 and actoS1. This is also indicated by the observation that the amplitude of the fluorescence signal is slightly larger with actoS1 than with S1, particularly at low ATP concentrations. This suggests that the fast phase is actually slower with actoS1 than with S1. With S1 the fast and slow phases are reasonably well separated at moderate and high ATP concentrations, but with actoS1 the slower fast phase contributes more to the observed signal, which therefore has a larger amplitude and appears faster.

At both temperatures the light-scattering signal is faster than the observed fluorescence signal, but since the fluorescence signal is partly due to the cleavage step, this is not a very meaningful comparison. At 1 °C the two fluorescence phases can be resolved, and under these conditions the fast fluorescence phase is clearly slower than light scattering at all ATP concentrations. At 20 °C the fast phase with ATP is too fast to measure accurately, but the apparent second-order rate constant measured at low ATP concentrations is slower by a factor of 2 when measured by fluorescence than by light scattering (Table 1). It appears therefore that the fast fluorescence phase is

Fig. 5. Concentration-dependence of the fluorescence change for ATP and ATP γ S binding to actoS1 at 20 °C

Conditions as Fig. 4. $\circ$, ATP; $\bullet$, ATP γ S; $\blacktriangle$, ATP γ S light scattering. The curve through the ATP γ S rates is the best fit to eqn. (1) for the light-scattering and fluorescence rates combined, with K_4 1000 M $^{-1}$, k_{+5} 380 s $^{-1}$. The curve through the ATP rates is drawn by hand, and the initial slope gives a value for the second-order rate constant of 1.5×10^6 M $^{-1}$ ·s $^{-1}$. The broken line indicates the rate of light scattering with ATP where the second-order rate constant = 2.5×10^6 M $^{-1}$ ·s $^{-1}$.

slower than dissociation at both temperatures, and so occurs on S1 alone.

DISCUSSION

The slow fluorescence phase

At high ATP concentrations at 20 °C the observed fluorescence change with both S1 and actoS1 is due almost entirely to the slow phase, since the fast step is complete within the dead time. The observed rate is similar to the rate of the phosphate burst and can be attributed to the cleavage step in all cases. Under the conditions used, complete dissociation takes place, and the cleavage step is the same starting from S1 or actoS1, in agreement with numerous previous studies. With ATP γ S the cleavage step is far too slow to be observed on these timescales, and only the fast phase is seen. It would be of

Fig. 6. Concentration-dependence of the fluorescence and light-scattering change for ATP and ATP γ S binding to actoS1 at 1 °C

Conditions as Fig. 4. $\triangle$, ATP (slow phase); $\circ$, ATP (fast phase); $\bullet$, ATP γ S fluorescence; $\blacktriangle$, ATP γ S light scattering. The curve through the ATP γ S rates is the best fit to eqn. (1) for the light-scattering and fluorescence data combined, with K_4 1900 M $^{-1}$, k_{+5} 110 s $^{-1}$. The curve through the rates of the ATP slow phase is drawn by hand, and the curve through the rates of the ATP fast phase is the best fit to eqn. (1) with K_4 2500 M $^{-1}$, k_{+5} 120 s $^{-1}$. The broken line indicates the rate of light scattering with ATP where the second-order rate constant = 5.6×10^6 M $^{-1}$ ·s $^{-1}$.

interest to know the size of the fluorescence enhancement associated with ATP γ S cleavage, but this is not possible as the cleavage step with ATP γ S is not distinguishable from the subsequent product release steps, and so its fluorescence signal cannot be resolved (Bagshaw *et al.*, 1972).

The rate of the fluorescence change at high nucleotide concentration is therefore due to different events with ATP and ATP γ S, and indeed the maximum rates with ATP γ S are about four times faster than with ATP at both temperatures (Figs. 2 and 3). The apparent similarity of these rates at pH 8.0, 23 °C observed by Bagshaw *et al.* (1974) is therefore coincidental. It has previously been shown that the similarity of the fluorescence enhancements observed when ATP and ADP bind to S1 at 23 °C,

0.1 M-KCl is also coincidental (Trybus & Taylor, 1982).

The fast fluorescence phase

The rate of the fast fluorescence phase with ATP is more difficult to measure. However, when ATP binds to actoS1 the apparent second order rate constant is slower when measured by fluorescence than by light scattering, and at 1 °C the maximum rate of the fast fluorescence phase is clearly slower than the maximum rate of actin dissociation. The fast fluorescence phase is therefore slower than dissociation, and so represents a transition between S1·ATP states. The simplest model that is compatible with these fluorescence data is a modified form of eqn. (1):

This is similar to the model proposed by Biosca *et al.* (1984) to account for their cold chase data. The assignment of observed rate constants to the steps in this equation is listed in Table 2.

This is different from the findings of Johnson & Taylor (1978). These authors found a single-phase fluorescence enhancement with actoS1 at 10 mM-KCl, 5–20 °C corresponding to cleavage (our slow phase) with a lag corresponding to actoS1 dissociation. Since they also

found a fluorescence enhancement on actoS1 association, they concluded that there was a fast fluorescence phase due to ATP binding, but it was cancelled out during the dissociation of actoS1. We can find no fluorescence change with actoS1 association, nor at the same rate as the ATP-mediated dissociation of actoS1, but we do observe a fast fluorescence change due to ATP binding. The reason for these differences is not known, although fluorescence measurements are very sensitive to experimental technique. Very large light-scattering changes accompany actoS1 association and dissociation, and any scattering of the incident beam which passes the emission filters would cause an apparent fluorescence change. We have examined the binding of actin to S1 in an SLM digital fluorimeter, and find that actoS1 has a slightly

smaller fluorescence signal than the sum of the individual spectra, the decrease being greater with higher concentrations of protein. This is presumably a result of the increase in light scattering on the formation of the actoS1 complex, which reduces the intensity of the incident beam, and so results in an apparently smaller fluorescence signal. We conclude that actoS1 has an intrinsic fluorescence similar to that of S1.

In relation to eqn. (2), it is of interest to consider the work of Rosenfeld & Taylor (1984) who used the fluorescent ATP analogue ϵ ATP to dissociate actoS1. These authors observed a nucleotide fluorescence change with a slower rate than light scattering, but they concluded that the rates were probably the same within experimental error. ϵ ATP has a slower cleavage rate than ATP, so the cleavage step was not contributing to this fluorescence signal, but the small difference in fluorescence and light-scattering rates could be explained if the observed fluorescence change was reporting an additional fast step following dissociation (such as step 7 in eqn. 2). The results of Rosenfeld & Taylor (1984) with ϵ ATP therefore appear to be similar to our results with ATP γ S. The fluorescence signal with ATP γ S has the same apparent rate as light scattering under normal conditions, but at sub-zero temperatures the fluorescence change clearly takes place after dissociation.

The model in eqn. (2) can adequately explain the fluorescence results. It is also compatible with the results of Johnson & Taylor (1978) if actoS1 has an enhanced fluorescence. The fast fluorescence enhancement on nucleotide binding to actoS1 can be explained in all cases by a fast binary isomerization (step 7), the observed rate of which is controlled by k_{+5} or k_{+7} (or a combination of the two), whichever is slower.

The mechanism of S1*ATP production from S1 and actoS1

There is general agreement that in 0.1 M-KCl S1*ATP is a common intermediate in the S1 and actoS1 pathways, since the cleavage step is common to both. Furthermore,

Table 2. Binding constants, K_d , and forward rate constants, k_{+i} , of the early steps of the S1 and actoS1 ATPase when described by eqn. (2)

All values are from this work except: ^aBagshaw *et al.* (1972), 20 °C, 0.1 M-KCl, pH 8; ^bGoody & Mannherz (1975), 4 °C, 10 mM-KCl, pH 8; ^cMillar & Geeves (1983), 0.1 M-KCl, pH 7. The binding constants of ATP and ATP γ S for actoS1 (K_d) are obtained from the light scattering data. The rate of the actin dissociation step (k_{+6}) is very fast so that the maximum observed rate of light scattering is governed by the preceding isomerization, k_{+5} (Millar & Geeves, 1983). With ATP γ S the rate of the fluorescence step (k_{+7}) is faster than k_{+5} , so the fluorescence change is observed at the same rate as light scattering. With ATP the rate of the fluorescence step (k_{+7}) is slower than k_{+5} , so the fluorescence can be observed as a slower event.

Constant	ATP		ATP γ S	
	20 °C	1 °C	20 °C	1 °C
K_1 (M ⁻¹)	500	800	~ 4500	~ 1800
k_{+2} (s ⁻¹)	> 1000	> 120	~ 340	~ 20
$k_{+3} + k_{-3}$ (s ⁻¹)	100	6	0.2 ^a	0.01 ^b
K_4 (M ⁻¹)	500	800	1000	1900
k_{+5} (s ⁻¹)	5000 ^c	700 ^c	400	100
k_{+6} (s ⁻¹)	> 700	> 700	> 400	> 100
k_{+7} (s ⁻¹)	> 1000	120	> 400	> 100

the fluorescence of this state, indicated by the amplitude of the fast fluorescence change, shows a common temperature-dependence. With both ATP and ATP γ S, and with both S1 and actoS1, the amplitude is reduced from 9 or 10 % at 20 °C to 4 % at 1 °C. This suggests that the structure of S1*ATP is different at 1 °C, with a lower fluorescence enhancement than at 20 °C. This is consistent with the general postulate that S1 can exist in one of two distinct conformations depending on temperature or bound nucleotide (Shriver, 1986).

The finding that the fast fluorescence transition with actoS1 occurs after actin dissociation means that there must be an additional S1·ATP intermediate on the actoS1 ATPase pathway prior to the common S1*ATP state. This intermediate is designated S1†ATP in eqn. (2) to indicate that it is non-fluorescent but not necessarily the same conformation as native S1. Two alternative routes (steps 2 and 2') are indicated in eqn. (2), depending on whether or not S1†ATP is also on the S1 ATPase pathway. With the ATP γ S-mediated dissociation of actoS1 at temperatures greater than 0 °C, the fluorescence change (step 7) is observed at the same rate as dissociation. This in turn is controlled by the ternary isomerization (step 5), so k_{+7} must be faster than k_{+5} (see Table 2). Since the observed fluorescence rate with actoS1 (controlled by k_{+5}) is faster than the observed fluorescence rate with S1 alone (controlled by k_{+2} or $k_{+2'}$), then k_{+7} must also be faster than k_{+2} or $k_{+2'}$. With ATP γ S therefore, the S1 pathway could possibly include step 7 (i.e. it would be steps 1, 2', 7 and 3), but on the available evidence it is not possible to say definitely whether S1†ATP γ S is an S1 intermediate or not.

The situation is more complicated with ATP, so it is worth reiterating two features of the results. Firstly, there is no sign of a fast fluorescence phase at 1 °C with S1 alone, although both phases are clearly seen with actoS1 at the same temperature, and Johnson & Taylor (1978) found evidence for a fast phase (with rate of 300–400 s⁻¹) at 5 °C. This suggests that if there is a fast fluorescence phase with S1 at 1 °C, then it must be faster than the rate of 120 s⁻¹ found for actoS1, or it would be seen. Secondly the observed fluorescence signal with actoS1 at 20 °C is slightly faster than the observed signal with S1 alone at the same temperature. As explained in the results section, this would be explained if the fast component was faster with S1 than with actoS1. The evidence is not compelling, but at both temperatures it does suggest that the fast fluorescence phase (i.e. the rate of production of S1*ATP) is faster with S1 than with actoS1. This would mean that step 7 cannot be on the S1 pathway and the intermediate S1†ATP must be unique to the actoS1 pathway. The S1 ATPase pathway simply consists of steps 1, 2 and 3. However, this does not rule out the possibility of additional S1·ATP states on the S1 pathway.

In this context, it is of interest to consider the binding of the non-hydrolysable nucleotides ADP and ADP[NH]P to S1. These have been shown to have biphasic fluorescence enhancements, which have been correlated with the two fluorescence enhancements of ATP binding and cleavage (Trybus & Taylor, 1982). However it has also been suggested that the much larger fluorescence enhancement of ATP binding is due to its extra step, namely cleavage (Bagshaw *et al.*, 1974).

Indeed the similarity of the size of the total fluorescence enhancement of ADP and ADP[NH]P with the fast phase only of the ATP fluorescence signal was one argument for assigning this phase to ATP binding rather than to the cleavage step. Furthermore, the binding of ϵ ATP to S1 results in two nucleotide fluorescence phases, prior to cleavage (Garland & Cheung, 1979; Rosenfeld & Taylor, 1984). Taken together, these results suggest that a 'universal' model for nucleotide binding to S1 would involve two first-order transitions, followed by cleavage where appropriate. However it is debatable whether nucleotides with such different binding kinetics should have a common mechanism, and such a model is certainly more complicated than required for ATP binding.

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