Determination of the Association of Myosin Subfragment 1 with Actin in the Presence of ATP†

Paul D. Wagner* and Alan G. Weeds

ABSTRACT: The mechanism of the actomyosin ATPase is multistep involving cyclic attachment and detachment of myosin and F-actin. The fraction of myosin bound to actin in the presence of substrate gives information about the distribution of myosin between these states. We have determined the fraction of rabbit myosin subfragment 1 (S-1) bound to actin in the presence of ATP, using an ultracentrifuge to separate bound and free S-1. Estimates have also been made of the extent of binding from turbidity measurements. These experiments were carried out at a number of different actin concentrations, both at 0.5 and 20 °C and at 17 and 35 mM salt concentrations. The fraction of S-1 bound at a given actin concentration was compared with the percentage saturation of the actin-activated ATPase at the same actin concentration, in an attempt to determine whether the rate-limiting step in the ATPase mechanism occurs when the S-1 is bound to actin or involves a transition of S-1 in the unbound state. At 20 °C, there was a reasonably good correlation between the fraction of S-1 bound to actin and the percentage saturation of the actoS-1 ATPase, while at 0.5 °C the fraction bound was much less than the saturation of the actoS-1 ATPase. These results suggest that the rate-controlling processes are different at 20 and 0.5 °C. Another approach to the problem is to compare the maximal rate of ATP hydrolysis per mole of S-1 at infinite actin concentration with that expressed per mole of actin at infinite S-1 concentration. Under the latter conditions, one would expect the maximal turnover of ATP to be determined by the rate of product release from the actin·S-1·ADP·P_i complex, whereas, under the former, any slow step involving S-1 alone would become apparent. Our experiments showed that at 5 °C the rate of ATP hydrolysis per mole of actin was two to four times faster than that per mole of S-1, but at 20 °C the difference between these rates was only 30%, again suggesting differences in the rate-controlling processes as a function temperature. The results of the binding measurements and steady-state ATPase activities indicate that at 20 °C the rate of product release from the actin·S-1·ADP·P_i complex is similar to, if not the same as, the steady-state rate in the actoS-1 ATPase; thus, a large fraction of S-1 may exist in this form at high actin concentrations. At low temperatures, only a small fraction of the S-1 is complexed with actin even at very high actin concentrations.

Lymn & Taylor (1971) have proposed a biochemical model for the actomyosin ATPase cycle that can be correlated with the mechanical cycle of cross-bridge attachment in muscle contraction. This scheme involved dissociation of actomyosin by ATP, hydrolysis of the ATP by myosin to give a myosin–products complex, reassociation of this complex with actin, and finally release of products as ADP and inorganic phosphate. The model was based on a number of steady-state measurements, principally those of Eisenberg & Moos (1970) and rapid reaction experiments which showed that the dissociation of actomyosin by ATP occurred at a faster rate than ATP hydrolysis (Lymn & Taylor, 1971). They proposed that, at high actin concentration, the rate-limiting step in the cycle was the release of products from actomyosin, so that under conditions approaching the maximum velocity, V_{max}, most of the subfragment 1 (S-1)[1] should be associated with actin. Eisenberg and co-workers have tested this aspect of the model, by measuring the proportion of S-1 or heavy meromyosin bound to actin under conditions where V_{max} could be approached experimentally (Eisenberg et al., 1972; Eisenberg & Kielley, 1972; Fraser et al., 1975). They found that the proportion of S-1 bound was much less than that expected if product release was the rate-limiting step. Thus, they proposed that the rate-limiting step in the actomyosin ATPase cycle is a transition of unattached S-1 from a refractory state (one incapable of binding to actin) to a nonrefractory one. The conditions chosen by Eisenberg & Kielley (1972) were low ionic strength and low temperature. The low ionic strength ensured maximal binding of actin so that V_{max} could be approached experimentally, while at low temperatures, substrate was not depleted during the ultracentrifuge runs.

Recent rapid reaction experiments both on subfragment 1 and its actin complex have shown that a number of the rate constants in the hydrolytic mechanism are strongly affected by changes in temperature and ionic strength (White & Taylor, 1976; Taylor, 1977). Thus, it is possible that the results obtained for S-1 binding to actin at 0.5 °C and low ionic strength would not be reproduced under more physiological conditions of temperature and ionic strength. For this reason, we have undertaken comparative experiments to investigate the relationship between actin binding and the percentage saturation of the actoS-1 ATPase activity over a range of different conditions. The results of experiments carried out a 0.5 °C confirm the observations of Eisenberg & Kielley (1972), but at 20 °C there is good correlation between the extent of S-1 binding and the percentage saturation of the actin-activated ATPase.

Two methods have been used to estimate the maximal activation of the S-1 ATPase by actin. The normal approach is to measure the rate of hydrolysis by using a fixed S-1 concentration and increasing actin concentrations. Alternatively one can use a fixed actin concentration and increasing S-1 concentrations to obtain a value for V_{max} at infinite S-1. This value is thought to reflect the rate of product release from the actin·S-1 complex. According to the original Lymn–Taylor model, the rates at saturating actin and at saturating S-1 should be the same since product release is proposed as the rate-limiting step. (Throughout this paper we refer to rates extrapolated to "infinite" actin, though in relating our results

† From the MRC Laboratory of Molecular Biology, Hills Road, Cambridge, CB2 2QH, England. *Received November 16, 1978.* P.D.W. is supported by a fellowship from the Muscular Dystrophy Association of America.

[1] Abbreviations used: S-1, myosin subfragment 1; ATPase, adenosine triphosphatase.

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to the Lymn–Taylor model we appreciate that, at infinite actin concentration, there will be no dissociation of the actin·S-1 complex (Lymn, 1974). Eisenberg & Kielley (1972) found that at 0.5 °C the rate of ATP hydrolysis per mole of actin was two to five times that per mole of S-1, which suggests that a different and slower step may be rate limiting in the hydrolysis of ATP by S-1 at infinite actin concentration. This process, which may precede actin reassociation, has been termed the refractory state transition. In this study, we have repeated these experiments at both 5 and 20 °C. At the lower temperature, the results were similar to those of Eisenberg & Kielley (1972). However, at 20 °C the difference in the rate of ATP hydrolysis per mole of actin and per mole of S-1 was only about 30%.

These results indicate that at 20 °C the rate-limiting step in the actoS-1 ATPase may indeed be associated with product release as originally proposed by Lymn & Taylor (1971), and it is not essential to postulate a refractory state to account for the experimental observations at this temperature.

Materials and Methods

Fast twitch myosin was isolated from rabbit back and white hind leg muscles (Lowey et al., 1969). S-1 was prepared by a chymotryptic digestion of myosin in 0.1 M sodium chloride, 20 mM sodium phosphate (pH 7.0), 1 mM ethylenediaminetetraacetic acid, 1 mM dithiothreitol as described previously (Weeds & Taylor, 1975). Actin was prepared as described by Taylor & Weeds (1976). A molecular weight of 115 000 and an $E_{280}^{1\%}$ of 7.5 cm^{-1} were assumed for S-1 (Weeds & Pope, 1977) and for actin 42 000 and 11 cm^{-1} (Elzinga et al., 1973; West et al., 1967).

The actin activated ATPase activity of S-1 was measured in the presence of varying concentrations of F-actin. The rate of ATP hydrolysis was determined by quenching the reaction at various times in cold 10% trichloroacetic acid and measuring phosphate release by the method of Rockstein & Herron (1951). The rate of ATP hydrolysis by actoS-1 has a hyperbolic dependence on actin concentration (Eisenberg & Moos, 1970) and can be described by the following equation $v = V_{max}[actin]/(K_m + [actin])$, where v is the observed rate of ATP hydrolysis by actoS-1 less that by S-1 alone, V_{max} is the maximal rate of ATP hydrolysis, and K_m the concentration of actin required for half-maximal velocity (the apparent dissociation constant of actin from S-1 in the presence of ATP). Values for V_{max} and K_m were determined by extrapolation of plots of v against $v/[actin]$. Throughout the text, V_{max} has been expressed as a turnover rate, in terms of the moles of ATP hydrolyzed per mole of S-1 per s, by using a molecular weight of 115 000 for S-1. The results obtained from these assays were used for comparison with the turbidity and ultracentrifugation experiments performed under identical conditions. The rate of ATP hydrolysis at infinite S-1 concentration was compared with that at infinite actin concentration by using data obtained from pH-stat assays. Determination of V_{max} at infinite S-1 concentration was carried out in the same manner as V_{max} at infinite actin concentration, except that the F-actin concentration was fixed and the S-1 concentration varied. Because of technical difficulties encountered by using a pH-stat at 0.5 °C, experiments were carried out at 5 °C for the low-temperature study.

Subfragment 1 binding to actin in the presence of ATP was monitored by turbidity at 340 nm in a dual beam spectrophotometer with water-jacketed cuvette holders. The turbidities of actin and S-1 were determined separately in the assay buffer, and the sum of their values was used as a base line for subsequent measurements. (Binding of S-1 to actin will reduce the value of the base-line S-1 turbidity; however, since the turbidity of S-1 alone was only 0.001–0.002, a very small fraction of the total turbidity, no attempt was made to correct for this.) Actin was incubated in the assay solution containing ATP until the temperature had equilibrated and the turbidity reached a constant value. A maximum volume of 0.2 mL of S-1 was added to give a final volume of 2.4 mL in the cuvette. The solutions were mixed and turbidity changes monitored by using a recorder. After mixing, there was an increase in turbidity over the base-line value which remained constant for periods of 0.75–60.0 min depending on the rate of ATP hydrolysis, after which there was a further sharp rise in turbidity to a plateau representing the final associated state of S-1 with actin. If buffer alone was added, the turbidity measurements were those expected for dilution of the actin. The increased turbidity was attributed to S-1 binding to actin. The amplitude of this signal in the presence of ATP was compared with the value obtained when all the ATP had been hydrolyzed, and this ratio was used as an estimate of the proportion of S-1 bound to actin in the presence of substrate under various conditions:

$$\% \text{ S-1 bound to actin in ATP} = [[A_{340}(\text{S-1} + \text{actin} + \text{ATP}) - A_{340}(\text{S-1}) - A_{340}(\text{actin})]/[A_{340}(\text{S-1} + \text{actin} + \text{hydrolyzed ATP}) - A_{340}(\text{S-1}) - A_{340}(\text{actin})]] \times 100$$

(i.e., initial increase in turbidity/final increase in turbidity).

The binding of S-1 to actin in the presence of ATP was also measured by using a preparative ultracentrifuge to separate bound S-1 from free. S-1 was added to actin, which had been equilibrated with the assay buffer, and samples of 2 mL were immediately centrifuged at 120000g for 90 min. About 1.8 mL of the supernatant was carefully removed and the extent of ATP hydrolysis measured from the phosphate concentration. Samples of 0.2 or 0.4 mL, depending on the initial S-1 concentration, were freeze-dried and taken up in buffer containing sodium dodecyl sulfate prior to electrophoresis on 7.5% polyacrylamide slab gels by using a discontinuous Tris–glycine buffer system (Laemmli & Favre, 1973). Gels were stained with Coomassie Brilliant Blue as described previously (Weeds, 1976). The S-1 concentration in each sample was estimated by densitometry of the heavy chain band by using a Camag gel densitometer. Optical density measurements were found to be linear between 5 and 25 μg of S-1. Control samples of S-1 were centrifuged at the same time as the actin-containing samples, either in the absence of any additional protein or in the presence of 0.1 mg/mL bovine serum albumin. In both cases, about 90% of the S-1 remained in the supernatant under these conditions and this value was used as a reference for determining the fraction of S-1 bound to actin. Duplicate control samples usually varied by about 5%. S-1 concentrations of about 0.35 μM were used in these experiments to minimize the rate at which ATP was hydrolyzed and, hence, to ensure that excess substrate remained when centrifugation was completed. Experiments carried out under conditions where all the ATP had been hydrolyzed showed that over 95% of the S-1 sedimented with the actin.

Results

Binding Studies at 20 °C. The interaction of S-1 with F-actin in the presence of ATP was monitored by turbidity changes. Two ionic conditions were chosen: (i) 3.0 mM disodium ATP, 3.3 mM $MgCl_2$, 1 mM KCl, 3 mM imidazole hydrochloride adjusted to pH 7.0 (I = 0.018; termed "low salt"); (ii) 5.0 mM disodium ATP, 5.5 mM $MgCl_2$, 3.0 mM KCl, 10 mM imidazole hydrochloride adjusted to pH 7.0 (I = 0.035; termed "high salt"). The low salt conditions were

Table I: Actin Activated ATPase Activities of S-1[a]

	20 °C		0.5 °C	
	V_{max} (s^{-1})	K_m (μM)	V_{max} (s^{-1})	K_m (μM)
low salt	11	19	0.35	4.1
high salt	13	64	0.52	28

[a] The conditions are given in the text. The S-1 concentration was fixed at 0.26 μM for the 20 °C assays and at 2.6 μM for the 0.5 °C assays and the actin concentration varied. In all cases, data were collected at actin concentrations above the K_m.

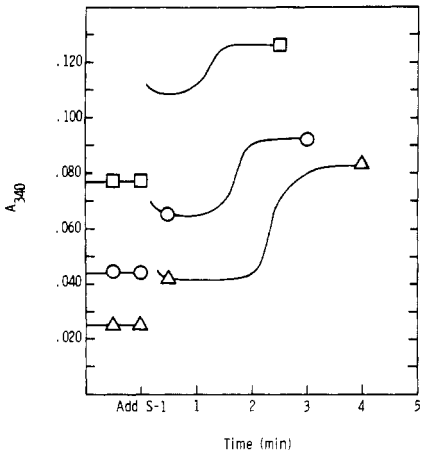


FIGURE 1: Turbidity changes of actoS-1 at 20 °C, low salt, and varying actin concentrations. The conditions are given in the text. The S-1 concentration was 4.30 μM, and the actin concentrations were 11 μM (△), 23 μM (○), and 46 μM (□). Turbidity measurements were monitored continuously and the symbols are used to delineate different experiments. The base line is the sum of the turbidities of actin alone and S-1 alone.

similar to those of Eisenberg et al. (1972), while the high salt conditions were chosen to suppress the differences in actin-activated ATPase activities between the two S-1 isoenzymes (unpublished observations). Values for V_{max} and K_m for the actin-activated ATPase activities under the four sets of conditions were determined from Eadie–Hofstee plots and these are given in Table I. These values were used to calculate the percentage saturation of the actin-activated ATPase activity at the various actin concentrations used in Tables II–V. Table I confirms the observations made by others that K_m is very sensitive to ionic strength, while V_{max} is much less sensitive, but there is a marked temperature dependence of the maximum turnover rate.

The change of turbidity on mixing S-1 with actin in the presence of ATP was examined initially at low salt and 20 °C. There was an immediate increase in turbidity which declined slightly to a plateau, the duration and amplitude of which were dependent on actin and S-1 concentrations. (This decline in turbidity was due to bubbles introduced during the mixing process.) When all the ATP had been hydrolyzed, the turbidity rose quickly to a second plateau (Figure 1). Occasionally at high actin concentrations there was a slow upward drift in the final turbidity which occurred at 20 °C but not at 0.5 °C. In these instances, the value at the top of the fast rise was used to estimate the increase in turbidity for the formation of the actin·S-1 complex. The size of the initial increase in turbidity was dependent on actin concentration as shown in Figure 1. This initial increase in turbidity was compared with the final increase in turbidity which occurred after exhaustion of the ATP. As shown in Table II, there was good correlation between this percentage value, which is an estimate of the extent of S-1 binding to actin in the presence

Table II: Interactions of S-1 and Actin at 20 °C and Low Salt[a]

actin (μM)	% saturation of actoS-1 ATPase[b]	% initial excess turbidity[c] A	B	C	% S-1 bound to actin in the ultracentrifuge[d]
56	75		80	73	
46	71		66		74 (7)
28	60		52		
23	55		44		54 (5)
14	42	25	33	34	
11	37	32	29	31	44 (6)

[a] The conditions used are given in the text. [b] Calculated from the K_m in Table I. [c] In A, B, and C, the S-1 concentrations were 8.60, 4.30, and 2.15 μM, respectively. The values given are averages of three or four separate determinations. [d] The S-1 concentration was 0.34 μM. The values given are averages of six runs and the numbers in parentheses are their standard deviations.

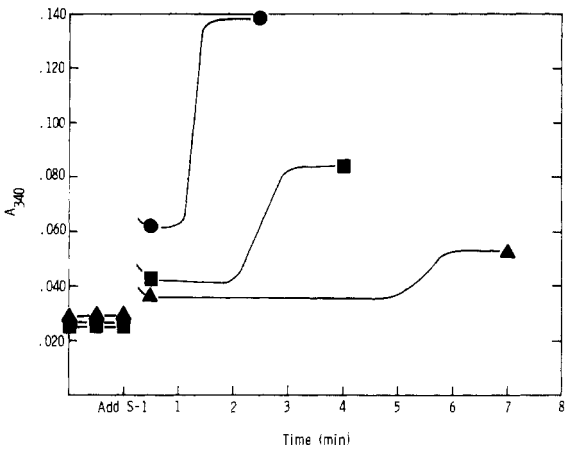


FIGURE 2: Turbidity changes of actoS-1 at 20 °C, low salt, and varying S-1 concentrations. The conditions are given in the text. The actin concentration was 11 μM, and the S-1 concentrations were 2.15 μM (▲), 4.30 μM (■), and 8.60 μM (●). The base line is the sum of the turbidities of actin alone and S-1 alone.

of ATP, and the percentage saturation of the actin-activated ATPase activity at the same actin concentration.

While the size of the initial increase in turbidity for a fixed S-1 concentration showed a marked dependence on actin concentration, the final increase in turbidity attained after depletion of the ATP was relatively constant for different actin concentrations. This was because at all times the actin concentration exceeded that of S-1. Since the affinity of actin for S-1 in the absence of ATP is high, all the S-1 will be bound even at the lowest actin concentrations used. However, in the presence of ATP, the affinity is much weaker and, hence, the extent of binding is dependent on the actin concentration. If, on the other hand, the actin concentration was kept constant, both the initial and final increases in turbidity showed marked dependence on the S-1 concentration used; however, the ratio of these two values, defined as percentage initial excess turbidity, remained relatively constant. For example, as shown in Figure 2 at 11 μM actin, 29% of the S-1 was bound at 0.25 mg/mL S-1, 28% at 0.5 mg/mL, and 31% at 1.0 mg/mL S-1. There was about a 15% variation in the value for final excess turbidity per mg/mL of S-1 for the different S-1 and actin concentrations used.

In principle, a more direct method for determining the extent of S-1 binding to F-actin is to use the ultracentrifuge to separate the complex from free S-1. Concentrations of S-1 were chosen such that the ATP was not completely hydrolyzed within the time necessary to sediment the complex. The actin concentrations were the same as those used in the turbidimetric

Table III: Interactions of S-1 and Actin at 20 °C and High Salt[a]

actin (μM)	% saturation of actoS-1 ATPase[b]	% initial excess turbidity[c] A	% initial excess turbidity[c] B	% S-1 bound to actin in the ultracentrifuge[d]
93	59	63	67	56 (6)
46	42	37	34	45 (5)
23	26	21	23	30 (9)
12	16	12	13	

[a] The conditions are given in the text. [b] Calculated from the K_m in Table I. [c] In A, the S-1 concentration was 8.60 μM and in B, 4.3 μM. The values given are averages of at least four separate determinations. [d] The S-1 concentration was 0.34 μM. The values given are averages of six runs and the numbers in parentheses are their standard deviations.

Table IV: Interactions of S-1 and Actin at 0.5 °C and Low Salt[a]

actin (μM)	% saturation of actoS-1 ATPase[b]	% initial excess turbidity[c] A	% initial excess turbidity[c] B	% S-1 bound to actin in the ultracentrifuge[d]
46	92	45		59 (2)
23	85	29		55 (3)
12	75	18	21	34 (8)
6	60		14	24 (5)

[a] The conditions are given in the text. [b] Calculated from the K_m in Table I. [c] In A, the S-1 concentration was 8.60 μM and in B, 4.30 μM. The values given are averages of at least four separate determinations. [d] The S-1 concentration was 0.34 μM. The values given are averages of six runs and the numbers in parentheses are their standard deviations.

Table V: Interactions of S-1 and Actin at 0.5 °C and High Salt[a]

actin (μM)	% saturation of actoS-1 ATPase[b]	% initial excess turbidity[c] A	% initial excess turbidity[c] B	% S-1 bound to actin in the ultracentrifuge[d]
92	76	44		41 (5)
46	62	26	33	28 (5)
23	45	9		24 (5)

[a] The conditions used are given in the text. [b] Calculated from the K_m in Table I. [c] In A, the S-1 concentration was 8.60 μM and in B, 4.30 μM. The values given are averages of at least four separate determinations. [d] The S-1 concentration was 0.34 μM. The values given are averages of six runs and the numbers in parentheses are their standard deviations.

assays. At an S-1 concentration of 0.04 mg/mL, the maximum rate of hydrolysis of ATP at infinite actin concentration is about 0.23 μmol min^{-1} mL^{-1}, which means that substrate will be exhausted after 13 min. The extent of hydrolysis was determined in each case by measurement of the inorganic phosphate concentration in the supernatant after centrifugation. If less than 0.7 mM ATP remained, the data were ignored. Some of the results are given in Table II. There is reasonable agreement between the percentage S-1 bound to actin as measured by this technique and that estimated from the turbidimetric assays.

To check that the extent of binding was not affected by substrate depletion, we carried out experiments at lower S-1 concentrations. Halving the S-1 concentration did not affect the percentage of S-1 bound except at the highest actin concentration (46 μM), where there was a 10% reduction in the proportion bound. This lower value might reflect an altered affinity of the S-1 for actin or could be an indication that ATP was depleted near the bottom of the centrifuge tube at the higher S-1 concentration.

Similar experiments were carried out at high salt and 20 °C. Once again the percentage initial excess turbidity was dependent on actin concentration, and values obtained correlated reasonably well with the percentage saturation of the actin-activated ATPase activity under the same conditions (Table III). Because of the higher K_m value at the increased ionic strength, at any given actin concentration, the increase in turbidity in the presence of ATP was less than that observed at the lower ionic strength. The percentage of S-1 bound to actin as measured with the preparative ultracentrifuge also closely followed the saturation of the actin-activated ATPase. Depletion of substrate was less of a problem at this higher ionic strength, both because the initial ATP concentration was higher and the extent of saturation of the actoS-1 ATPase was lower. Even at the highest concentration of actin used, there was more than 2 mM ATP remaining after centrifugation was completed.

Binding Studies at 0.5 °C. Eisenberg et al. (1972) reported that at 0.5 °C and low ionic strength only a small fraction of S-1 bound to F-actin in the presence of ATP, which is in marked contrast to the results obtained here at 20 °C. Therefore, these experiments were repeated under the same salt conditions but at 0.5 °C. At low salt, addition of S-1 to actin under conditions where the saturation of the ATPase activity was about 60% gave only a slight increase in turbidity compared with the final value obtained after the all ATP had been hydrolyzed; the initial increase in turbidity was about 14% of the final turbidity (Table IV). At higher actin concentrations where the ATPase would be 92% of the maximal value, the initial increase in turbidity reached 45% of the final value after all the ATP was hydrolyzed. As was the case at 20 °C, the percentage increase was the same for several S-1 concentrations at any fixed actin concentration, but there was more variation in final turbidity level attained after the ATP had been hydrolyzed when the amplitude of this measurement was normalized to S-1 concentration.

The extent of binding was also determined in the preparative ultracentrifuge. The results, although somewhat higher than those obtained from turbidity measurements, were significantly lower than percentage saturation of the ATPase activity at the same actin concentration. Because of the slow turnover of ATP at this temperature, there was about 2 mM ATP left in the supernatant after centrifugation.

Experiments were also carried out at high ionic strength and 0.5 °C. Here the determination of turbidity at the highest actin concentrations was more difficult because of bubbles present in the viscous solutions, but these rose to the surface of the cuvettes if the solutions were left sufficiently long. Results are given in Table V and they are consistent with those obtained at low ionic strength. Thus experiments carried out at 0.5 °C confirm the earlier observations (Eisenberg et al., 1972; Eisenberg & Kielley, 1972; Fraser et al., 1975) that the extent of binding of S-1 to actin in the presence of ATP is much less than that expected if the steady-state rate of ATPase activity is controlled by release of products from the actin·S-1 complex.

ATPase Activities. The maximal rate of actoS-1 ATPase can be determined in two ways: (1) by fixing the S-1 concentration and increasing the actin concentration or (2) using a fixed actin concentration and increasing the S-1 concentration, and in either case correcting for the slow hydrolysis of ATP by S-1 alone. In both cases, the varied species is treated as the "substrate" in conventional Michaelis–Menton kinetic analysis and the maximal velocity determined by extrapolation to infinite "substrate" (Eisenberg & Moos, 1970). (The correction applied for the turnover of ATP by

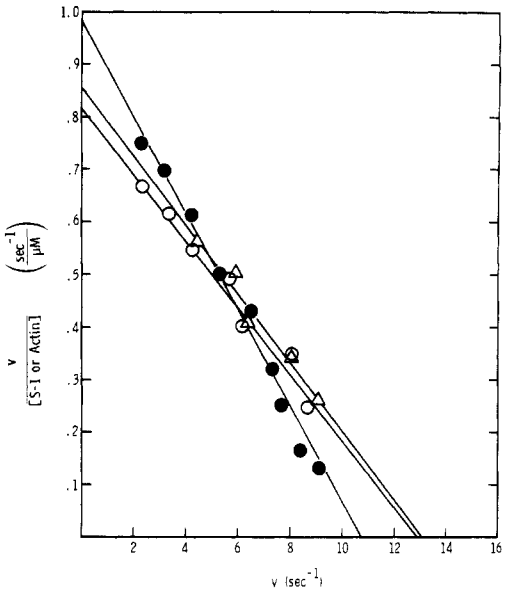


FIGURE 3: ActoS-1 ATPase activity at 20 °C and pH 7. The conditions are given in the text. In one instance, the S-1 concentration was fixed at 0.77 μM and the actin varied (●). In the other two, the S-1 concentration was varied and the actin fixed at 4.0 μM (○) and 6.0 μM (△).

S-1 alone is very small under conditions where the S-1 concentration is fixed, but under the second set of conditions this assumed a more significant value but is never greater than 10% of the observed turnover rate under the conditions of these experiments, since the activation of the ATPase activity by actin is at least 200-fold). Values for V_{max} and K_m were determined from Eadie–Hofstee plots at both 5 and 20 °C under the following conditions: 3 mM disodium ATP, 3 mM $MgCl_2$, 6 mM KCl, pH 7.0. At 5 °C, V_{max} expressed per mole of S-1 at infinite [actin] was 1.0 s^{-1} (K_m = 2.3 μM actin), while the corresponding value expressed per mole of actin at infinite [S-1] was 3.0 s^{-1} (K_m = 17 μM S-1) by using an actin concentration of 2 μM. In a further experiment with 4 μM actin, V_{max} was 2.0 s^{-1} and K_m 13 μM S-1. These results are consistent with those observed at 0.5 °C by Eisenberg & Kielley (1972). However, as shown in Figure 3, at 20 °C the differences between these two extrapolated values were much smaller. This experiment has been carried out by using four different S-1 and actin preparations. In all cases, the rate per mole of actin was greater than that expressed per mole of S-1, but the difference between the two extrapolated values varied between 10% and 50%, not by a factor of 2–3 as found at 5 °C. Furthermore, unlike the results obtained at 5 °C, there was no obvious difference in the rate constants expressed per mole of actin at infinite S-1 concentration whether 2, 4, or 6 μM actin was used. The average values expressed per mole of S-1 at infinite actin were V_{max} = 12 ± 3 s^{-1} and K_m = 12 ± 1 μM actin, while those expressed per mole of actin at infinite S-1 were V_{max} = 14 ± 2 s^{-1} and K_m ± 3 μM S-1.[2]

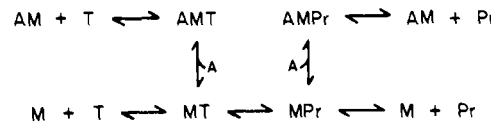
Discussion

Before discussing these results in relation to any kinetic model, it is important to evaluate the experiments themselves. The first method chosen to estimate S-1 binding to actin in the presence of ATP was turbidimetric. This method has the advantage of being rapid and easy to perform and is, therefore, well suited to conditions where substrate availability is limited by the requirement of maintaining low ionic strength. The disadvantage of the method is that the turbidity increase associated with S-1 binding to actin even at low actin concentrations is not linear, showing slight upward curvature (Chock et al., 1976; White & Taylor, 1976). One way of examining the quality of the data presented here is to normalize the final excess turbidity to amount of S-1 added. At 20 °C, over a fourfold range in S-1 concentration and a sevenfold range in actin concentration, this value varied by only about 15%. There was no consistent trend in this variation and it appeared to be random. At 0.5 °C for a fixed actin and varying S-1, the error was also about 15%. Errors of this magnitude were sufficiently large to obscure those small deviations from linearity which have been reported by others (Chock et al., 1976; White & Taylor, 1976).

Because of the nonlinearity of the turbidity measurements and possible reservations about interpreting turbidimetric experiments in terms of S-1 binding, it was important to use another method to assess binding. Ultracentrifugation provides a direct measure of S-1 binding to actin. The analytical ultracentrifuge has been used by a number of investigators to measure the association constant of S-1 and actin in the absence of ATP. In addition, the preparative ultracentrifuge has been used by Takeuchi & Tonomura (1971) and by Marston & Weber (1975) to determine the same binding constant. Both methods make the same assumptions: (1) the F-actin sediments as a sharp boundary so that the equilibrium is not disturbed by changes in actin concentration; (2) the equilibrium is not affected by hydrostatic pressure during the centrifugation. Marston & Weber (1975) found little difference in the equilibrium constant whether the centrifugation was carried out at 50000*g* or 140000*g*, indicating that this latter assumption was probably valid. Eisenberg & Kielley (1972) have measured the binding of S1 to actin in the presence of ATP at 0.5 °C by using the analytical ultracentrifuge. However, this technique is not suitable at 20 °C because substrate hydrolysis is too rapid at S-1 concentrations required for detection even by the UV scanner. By using preparative ultracentrifugation, it is possible to determine S-1 concentrations in the supernatant at much lower levels if the samples are applied to polyacrylamide gels and the protein is estimated by densitometry. Thus, experiments were carried out at about 0.35 μM S-1, but, even at this protein concentration, substrate would be exhausted in a relatively short time. For example, at 20 °C and an actin concentration to give 75% V_{max} (the most stringent conditions used), substrate will be exhausted after 17 min. Although centrifugation was carried out for 90 min, actin should pellet in much shorter times than this. Using a sedimentation coefficient of 200 S, we estimated that actin should sediment to the bottom of the centrifuge tube in about 10 min. Longer centrifugation times were used to ensure the formation of a tight pellet which would not be disturbed when the supernatant was removed. However, it is obvious that ATP utilization will not be the same throughout the solution as the actin sediments and there may be depletion of substrate at the bottom of the tube. Thus, even if the average ATP concentration measured at the end of centrifugation was sufficient to dissociate S-1 from actin, the effective concentration at the bottom of the tube might be much less. This would result in an overestimate of the amount of S-1 bound. One way to test whether such depletion of ATP might be occurring is to measure the fraction of S-1 bound at a given actin concentration by using two different S-1

[2] After completion of these ATPase assays, Marston (1978) published results of similar experiments performed at 25 °C. He also found only a small difference in the rates of ATP hydrolysis per mole of actin at infinite S-1 and per mole of S-1 at infinite actin.

Scheme I: Lymn and Taylor Scheme



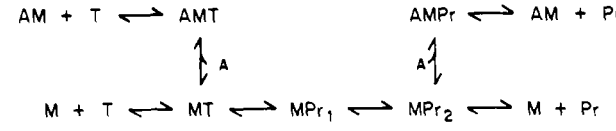
concentrations. With one exception, all experiments of this nature showed no significant variation in the amount of S-1 bound; the exception was an experiment carried out at low ionic strength and 20 °C by using an actin concentration which gave 70% saturation of the ATPase activity: halving the S-1 concentration reduced the fraction of bound S-1 from 75% to 65%. Although this result might indicate an overestimate of S-1 binding at the highest actin concentrations used, the agreement between the two methods under all four sets of conditions gave us confidence in the validity of these results.

While the turbidity and ultracentrifugation experiments were performed with a large number of S-1 and actin preparations, values for K_m and V_{max} were obtained from single sets of ATPase assays. As noted previously, there is some variation in K_m and V_{max} between S-1 and actin preparations. If these variations are large, the comparison of percentage saturation of the actoS-1 ATPase with the extent of binding would be invalid. By measurement of the time taken for all the ATP to be hydrolyzed in the turbidity assays (see Figures 1 and 2), it is possible to calculate the rate of hydrolysis in each experiment and from these data to estimate values for V_{max} and K_m under each set of conditions. While this method is not very accurate since the number of experimental points is low, the results obtained were with one exception within 15% of those obtained by direct ATPase measurements. These calculations add confidence to the validity of our comparisons.

The degree of binding observed in the presence of ATP should reflect the distribution of S-1 in the various kinetic states of the actomyosin ATPase cycle. There are currently two major schemes for the actomyosin ATPase cycle. The first was originally postulated by Lymn and Taylor in 1971. A simplified version can be written as shown in Scheme I, where A, M, T, and Pr represent actin, myosin, ATP, and ATP hydrolysis products, respectively. ATP binds to the actomyosin causing a rapid and complete dissociation of the myosin from the actin. Hydrolysis of the ATP occurs subsequently on the myosin alone. In the absence of actin, the release of products is very slow and, hence, this is the rate-limiting step. Actin binds to the myosin–products complex, and the rate of release of products from this ternary complex is faster than that from myosin alone, causing an increase in the overall rate of ATP hydrolysis. Since the rates of actomyosin dissociation and ATP cleavage are fast, Lymn & Taylor concluded that the rate-limiting step in the actomyosin ATPase was also release of products. In subsequent papers more emphasis has been put on the reversability of the steps involving actin binding but the basic scheme remains the same (White & Taylor, 1976; Sleep & Taylor, 1976). Eisenberg and co-workers have introduced an additional step to explain the low levels of association between actin and S-1 at high levels of saturation of the acto-S-1 ATPase (Scheme II).

The rate-limiting step in this scheme is the transition from one state of myosin–products complex to another (i.e., from a refractory to a nonrefractory state). Thus even at saturating actin concentrations, only a fraction of the S-1 will be bound to actin, the rest being trapped in the refractory state (Eisenberg et al., 1972; Fraser et al., 1975). More recently Chock et al. (1976) showed that the rate of actin reassociation with myosin–products was the same as the steady-state ATPase and

Scheme II: Refractory State Model



showed an identical hyperbolic dependence on actin concentration. If the reassociation were a simple bimolecular reaction, its rate would increase linearly with actin and not saturate at high actin concentration. However, the observed saturation is consistent with a two-step process, a slow unimolecular transition on the myosin alone, from the refractory to the nonrefractory state, followed by a rapid binding to actin (Chock et al., 1976). These experiments were performed at 5 °C and very low salt concentration. White & Taylor (1976) have also measured the rate of actin reassociation in a single turnover but over a much larger range of temperature and ionic strength. However, all their measurements were under conditions where the stimulation by actin varied linearly with actin concentration, i.e., well below K_m. They found that, under all conditions examined, the rate of reassociation was close to the observed rate of ATP hydrolysis and slower than that expected for a simple bimolecular reassociation, suggesting a more complicated mechanism. One explanation is similar to that given by Eisenberg and co-workers, namely, that a slow first-order reaction precedes actin binding. An alternate explanation is that actin binding is a rapid reversible equilibrium which is followed by a slow first-order process, e.g., product release. Taylor (1979) in a recent review discusses the various kinetic models. If the reversibility of actin reassociation is taken into account as well as the known reversibility of the ATP cleavage step (Bagshaw & Trentham, 1973; Taylor, 1977), it is possible to account for the low levels of actin S-1 association observed without postulating a refractory state.

The binding studies presented here at 0.5 °C agree reasonably well with those of Eisenberg and co-workers (Eisenberg & Kielley, 1972; Fraser et al., 1975). Using an analytical ultracentrifuge, they found that at 59% and 78% saturation of the actin activated ATPase only 16% and 22% of the S-1 was bound. In experiments reported here by using the preparative ultracentrifuge and similar saturations of the actin activated ATPase, 24% and 34% of the S-1 was bound to the actin. (The differences between these two numbers could reflect the type of S-1 used or some systematic error in either or both techniques.) At very high actin concentrations, about half of the S-1 is bound. The data are not sufficiently accurate to determine if this reflects an approach to saturation. At twice the ionic strength at 0.5 °C, there was still a large discrepancy between the saturation of the actin-activated ATPase and the fraction of S-1 bound to actin. However, the data seem to indicate that a slightly larger fraction is bound at the higher ionic strength. These results are consistent with the existence of a refractory state but, as discussed by Taylor (1979), simple reversibility of the various steps in the actin-activated ATPase could also account for this discrepancy.

Our results at 20 °C differed significantly from those at 0.5 °C. At the higher temperature and for both ionic strength values, the amount of S-1 bound follows closely the saturation of the actin-activated ATPase.[3] The simplest interpretation of these results is that the rate of hydrolysis is controlled by

[3] E. Eisenberg (personal communication) has measured the extent of actin S-1 binding at 15 °C in the presence of ATP using turbidity changes in the stopped-flow spectrophotometer. The extent of binding at this temperature is intermediate between that observed at 0.5 and 20 °C.

a process where actin and S-1 are associated, and this process might be product release. These results need not imply a different mechanism of ATP hydrolysis at the different temperatures but could simply reflect a different temperature dependence of individual steps in the same mechanism.

One means of analyzing the influence of processes subsequent to actin reassociation on the overall ATPase rate is to measure the rate of hydrolysis with increasing S-1 concentrations and extrapolate the data to infinite S-1. The rate observed under these conditions will be unaffected by slower first-order transitions of the refractory state type, because the concentration of the nonrefractory species will also approach the infinite and cannot be limiting in availability. This is in marked contrast to conditions where the actin concentration is extrapolated to infinity at a fixed S-1 concentration, because under these conditions the rate of any transition occurring on the isolated S-1 will control the availability of nonrefractory material. Thus at 5 °C, the rate of ATP hydrolysis expressed per mole of actin at infinite S-1 was two to four times faster than the rate expressed per mole of S-1 at infinite actin which is consistent with a refractory state model. These results are in agreement with those reported by Eisenberg & Kielley (1972) and are consistent with the actin binding data obtained at low temperature. However, the important question we have addressed concerns the validity of extrapolating these experimental conclusions to higher temperatures, and the results obtained at 20 °C show much closer agreement between the two extrapolated ATPase values. A difference of only 30% may well indicate that the two rates are the same since errors in the determination of protein concentration and possible denaturation can lead to variation of this magnitude. Both from these results and the binding experiments, it appears either that, at 20 °C, the rate of product release from the actin·S-1 complex limits the overall rate of ATP hydrolysis or that this rate is only marginally faster than the overall turnover rate so that a large fraction of the S-1 is associated with actin under these conditions. As discussed previously (Weeds, 1977), the importance of the refractory state concept is that it has focused attention on processes preceding actin reassociation in the actomyosin ATPase, in particular the reversibility of the cleavage step itself (Trentham, 1977; Taylor, 1977). In the past, kinetic schemes have been postulated in their simplest forms, leaving out considerations of reversibility except where this was clearly demonstrated. It is now becoming apparent that the reversibility of a number of other steps in the mechanism must be considered, which will not only make the overall scheme more complex but also may cast doubts on some of the assumptions arising from the Lymn–Taylor model (for example, that actomyosin dissociation occurs before ATP cleavage and, hence, ATP cleavage does not occur on the associated complex). Taylor (1979) has drawn attention to these problems in his recent review and experiments are already under way in a number of laboratories to test these more complex models.

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