Energetics and Mechanism of Actomyosin Adenosine Triphosphatase†

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ABSTRACT: Rate constants were determined for the reaction of actin with subfragment 1 (S1), S1–product complex, heavy meromyosin (HMM), and HMM–products complex for a range of temperatures, pH's, and ionic strengths. For actin concentrations up to 10 μM, the rate of reassociation of the product intermediate was equal to the rate of actomyosin subfragment 1 (acto-S1) or acto-HMM adenosine triphosphatase (ATPase). Therefore, under these conditions, the only important pathway for adenosine triphosphate hydrolysis is through the dissociation and recombination of S1 or HMM. The apparent rate constants for the association of S1 and S1–product with actin showed a similar large ionic strength dependence. The S1–product reaction had a large temperature dependence paralleling the rate of acto-S1 ATPase, while the reaction with S1 had a much smaller variation with temperature. The low value of the rate constant for the S1–product reaction and its relationship to the S1 reaction suggests that the apparent rate constant does not measure a simple second-order reaction. A plausible mechanism is a rapid equilibrium for the binding step, followed by a transition (product release) which increases the association constant. A refractory state could also reduce the apparent rate constant of recombination. An approximate assignment of equilibrium constants for the acto-S1 ATPase reaction was made based on the interpretation of the present evidence and equilibrium constants for the S1 ATPase.

The mechanism of actomyosin ATPase[1] is closely related to the events in the cross-bridge cycle of muscle contraction. The steady-state rate was shown to depend hyperbolically on actin concentration at constant myosin concentrations by Eisenberg and Moos (1968) and by extrapolation to infinite actin concentration they obtained a maximum rate of the proper magnitude to account for ATP turnover in muscle. The result is consistent with a simple association of the AM complex in determining the rate of hydrolysis but transient kinetic studies by Lymn and Taylor (1971) showed AM dissociation by ATP to be faster than the hydrolytic step. The following mechanism was proposed to account for these findings:

$$\begin{array}{ccccccc} \mathrm{AM} & \overset{(1)}{\rightleftharpoons} & \mathrm{AM{\cdot}ATP} & & \mathrm{AM{\cdot}Pr} & \overset{(5)}{\rightleftharpoons} & \mathrm{AM} + \mathrm{Pr} \\ {\scriptstyle(8)}\updownarrow & & \updownarrow{\scriptstyle(2)} & & \updownarrow{\scriptstyle(4)} & & \updownarrow{\scriptstyle(8)} \\ \mathrm{M} & \overset{(6)}{\longrightarrow} & \mathrm{M{\cdot}ATP} & \overset{(3)}{\rightleftharpoons} & \mathrm{M{\cdot}Pr} & \overset{(7)}{\longrightarrow} & \mathrm{M} + \mathrm{Pr} \end{array} \quad (1)$$

where M·Pr refers to a myosin–products intermediate state. Rate constants k_i and k_{-i} are assigned to each of the numbered steps.

It was assumed that the maximum rate obtained by extrapolation determined k_5, since $k_3 \gg k_5$. (At 20 °C, 50 mM KCl, pH 8, the values are ~150 and 10 s^{-1}, respectively.) While it was shown that actin displaces products from the M·Pr state, k_4 was not directly measured. A necessary property of the model is that k_4 determines the steady-state rate at a nonsaturating actin concentration. Preliminary measurements with an ATP regenerating system (Koretz et al., 1972) showed k_4 to be of the correct magnitude but a proper comparison was not made.

Further studies of myosin and actomyosin ATPase have made it necessary to reassess this simple scheme. The myosin ATPase mechanism is now known to proceed in six or seven steps, which greatly increases the number of possible pathways for the actomyosin cycle (Bagshaw et al., 1974; Bagshaw and Trentham, 1974; Koretz and Taylor, 1975; Chock and Eisenberg, 1974; Mannherz et al., 1974). Besides the states defined by kinetic studies of myosin, Eisenberg and Kielley (1972) have provided evidence for a "refractory state" that is a myosin state which does not combine with actin. The maximum rate could be determined by a transition of this state rather than by step 5. Lymn (1974 a,b) has also criticized the determination of k_5 by extrapolation to infinite actin concentration on theoretical grounds. Finally, Inoue et al. (1973) and Hozumi and Tawada (1974) have reported that k_4 is two to five times too small to account for actomyosin ATPase.

The sequence of states in dissociation was investigated by Sleep and Taylor (1976) in a study of the relative rates of dissociation, hydrolysis, and the conformation change monitored by fluorescence enhancement. It was shown that dissociation precedes the conformation change and the first dissociated state in the pathway is one in which ATP is tightly bound. This state may be distinct from the intermediates of the myosin pathway. Thus, these results did not alter one of the important properties of the Lymn–Taylor mechanism, since it was also found that the hydrolysis step follows the myosin conformation change and is at least 100 times slower than dissociation at low temperature.

The main purpose of the present study was to measure the rate of step 4 over a range of conditions of pH, ionic strength, and temperature in order to answer the question of whether it accounts satisfactorily for actomyosin ATPase activity. The rates of actin–myosin association and dissociation and the rates of ATP-induced dissociation were measured for a range of conditions to allow a comparison of the association–dissociation processes. Together with previous evidence on rates and equilibria, a preliminary assignment of equilibrium constants can be made for the states in the actomyosin cycle. While the question of interaction between heads cannot yet be answered, the evidence as applied to acto-S1 ATPase is a first step in

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[1] Abbreviations used are: ATPase, adenosine triphosphatase; ATP, adenosine triphosphate; HMM, heavy meromyosin; acto, actomyosin; S1, subfragment 1; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol; Nbs_2, 5,5′-dithiobis(2-nitrobenzoic acid).

determining whether the mechanism is energetically feasible as a basis for muscle contraction.

Materials and Methods

Actin was prepared by the method of Drabikowski and Gergely (1964). The pellets were resuspended as F-actin with a glass homogenizer in the appropriate buffer. Actin prepared in this way is free from tropomyosin and troponin. Contamination by free nucleotides was always less than 0.1 mol/mol of actin monomer. Myosin was prepared by the method of Perry (1955) from rabbit back and leg muscles. Initially, heavy meromyosin was prepared by tryptic digestion (Lowey and Cohen, 1962) but the actin activation of our preparations varied by as much as fivefold. Adjusting the pH or time of digestion did not reduce this variability. For most experiments, HMM was prepared by chymotryptic digestion (Weeds and Taylor, 1975). Chymotryptic HMM contained at least 90% intact heavy chains and the same proportions of the three light chains as the original myosin as determined by sodium dodecyl sulfate-acrylamide gel electrophoresis. Occasionally, this method produced HMM contaminated with 10–15% S1. S1 contamination was reduced by adjusting the digestion time to give a yield of approximately 40% of the theoretical value. The acto-HMM ATPase was $2.0 \pm 0.5\ s^{-1}$ per site under "standard conditions" (10 μM actin, 40 mM KCl; 20 °C, pH 8). For the experiments done with tryptic HMM, only preparations with an acto-HMM activity of $1.5\ s^{-1}$ or larger were used.

Subfragment 1 was prepared by digestion with papain (Lowey et al., 1969) or by the method of Weeds and Taylor using chymotrypsin. Chymotryptic S1 contained intact heavy chains of molecular weight approximately 90 000 and both alkali light chains, but lacked the Nbs_2 light chains. Papain S1 often has partially cleaved heavy chains but contains the three light chains if magnesium is present during digestion. The acto-S1 ATPase was $2.5 \pm 0.5\ s^{-1}$ for chymotryptic S1 and $1.5 \pm 0.5\ s^{-1}$ for papain S1 under standard conditions.

Molar protein concentrations were determined from the following extinction coefficients and molecular weights: S1, 0.77 cm^2/g at 280 nm and 115 000; HMM, 0.654 cm^2/g at 280 nm and 340 000; actin, 0.69 cm^2/g at 290 nm, 1.15 cm^2/g at 280 nm and 42 500. Steady-state turbidity measurements were made with a Cary Model 15 or a Unicam spectrophotometer at 400 nm. Rapid kinetic measurements were made using the transmitted beam of an Aminco Morrow stopflow spectrophotometer at 400 nm or from 90° light scattering using a stopflow spectrofluorimeter. Both methods give the same values for rate-constant measurements. The output signals were stored on a Tektronix 5103N oscilloscope and measurements were made from Polaroid photographs of the traces. The turbidity of S1 or HMM was generally negligible compared to actin. The G actin content was less than 10% based on sedimentation and it does not contribute to changes in turbidity. The change in voltage divided by initial voltage was 0.1 or less. Association reactions with one reactant in excess were fitted to the equation $\Delta V = \Delta V_f(1 - \exp(-\lambda t)$ by a semilog plot. ΔV is the increase in voltage relative to actin at time t and ΔV_f is the maximum voltage change. Dissociation reactions were fitted to $\Delta V = \Delta V_f \exp(-\lambda t)$. Calibration of the voltage, when required, was obtained by taking the voltage for actin alone as complete dissociation.

ATPase measurements were made by titrating the protons released by ATP hydrolysis with 10 mM NaOH in a Radiometer TTT1b pH-stat equipped with a thermostated cell and nitrogen bleed. Standard reaction conditions were 1 mM ATP, 2 mM $MgCl_2$, 40 mM KCl, 20 °C, pH 8.0, in a total volume of 5.0 ml.

Calibration of the titration gave a value of 1 mol of base consumed for the hydrolysis of 1.17 mol of ATP. This figure corresponds to a pK of 7.2 for the ionization of the phosphate produced in hydrolysis.

Interpretation of Rate Measurements. Association and dissociation reactions of AS1 and AHMM were studied by turbidity or light scattering and the results are interpreted using fairly simple kinetic schemes. It is necessary to state clearly the assumptions made in deriving kinetic equations and in using scattering as a measure of the degree of association of large molecules.

The association of S1 with actin is treated as the independent binding of S1 to G actin residues. On this assumption, the fraction of the sites occupied in an actin polymer of length j residues for any j is equal to the fraction α of the total G actin which is complexed with S1. The reaction can be written simply as

$$A + S1 \underset{k_{-s}}{\overset{k_s}{\rightleftharpoons}} AS1$$

where A and S1 refer to the molar concentrations of G actin and S1. With S1 in excess, the rate equation is $\alpha = \alpha_f[1 - \exp(-\lambda t)]$, where α_f is the final degree of association at equilibrium and $\lambda = k_s[S1] + k_{-s}$.

The association of HMM with actin shows the same kinetic behavior as S1. The reaction follows a pseudo-first-order rate equation for HMM in excess and the rate varies linearly with concentration. The reaction is treated as simple independent binding

$$A + HMM \underset{k_{-h}}{\overset{k_h}{\rightleftharpoons}} AHMM$$

and since the rate of dissociation is extremely small, the reaction is essentially irreversible. The assumption of independent binding ignores the possibility that, as the reaction goes to completion, single G actin sites will be left over which may bind 0 or 1 HMM. The fraction of single sites was calculated by treating the combinatorial problem as a random process. For a very long polymer, the fraction of single sites is $e^{-2} = 0.135$ and the value is slightly smaller for polymers of finite length. In view of the other sources of error, it seems reasonable to ignore this effect.

The two heads of HMM are generally assumed to be attached to adjacent G actin residues. The kinetic mechanism should specify the rate of attachment of the second head relative to the first and representing the binding as a single step process is equivalent to assuming the two heads bind simultaneously. The rate constant k_h is a composite rate constant, which describes the overall process, but it should be regarded as an experimental number defined by a one-step binding mechanism. The value of k_h relative to k_s can only be specified by a model which treats the two-step process explicitly.

The association of actin with S1-product and HMM-product states was measured by double-mixing experiments with actin residues in excess. The results satisfied a pseudo-first-order rate equation and can be represented by

$$S1{\cdot}Pr + A \overset{(4)}{\rightleftharpoons} AS1Pr \overset{(5)}{\rightleftharpoons} AS1 + Pr$$

The steps are numbered as in eq 1. The signal measures the associated states and the rate constant, defined by measurements over a range of actin concentration for which the rate is linear in actin concentration, defines an apparent second-

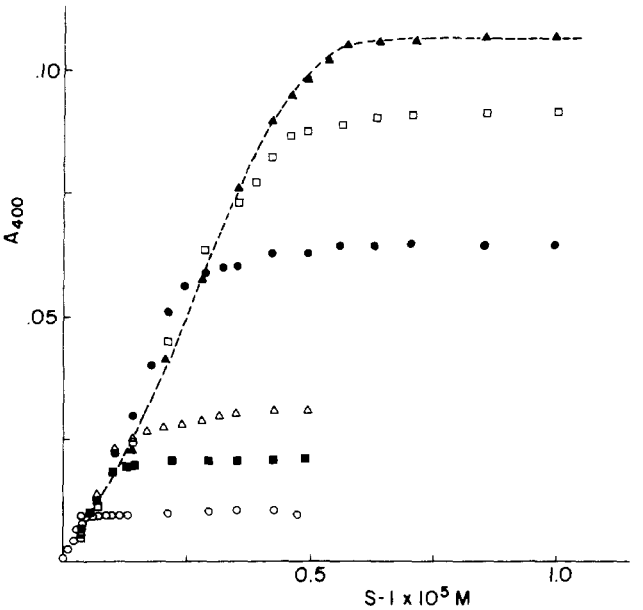


FIGURE 1: Turbidimetric titration of actin with S1. Actin concentration: 0.4 (○), 0.8 (■), 1.2 (△), 2.5 (●), 4.0 (□), 5.0 (▲) μM. Experimental conditions: 100 mM KCl, 1.0 mM $MgCl_2$, 10 mM Tris, pH 8.0, 20 °C.

order-rate constant k_4^a, where the measured rate, λ, is $\lambda = k_4^a(A)$ and actin concentration is expressed as G actin. The reaction occurs in at least two steps and the relation of k_4^a to the molecular rate constants, k_4, k_{-4}, and k_5, and to a possible refractory state will depend on the kinetic model.

The dissociation of AS1 or AHMM by ATP fitted a single exponential and the rate was proportional to ATP concentration. The reaction must take place in at least two steps,

$$AS1 + ATP \overset{(1)}{\rightleftharpoons} AS1\cdot ATP \overset{(2)}{\rightleftharpoons} A + S1\cdot ATP$$

$$AS1 = (AS1)_0 \exp(-\lambda t),\ \lambda = k_1^a[ATP]$$

The experiment defines an apparent second-order rate constant k_1^a for ATP binding, which is some combination of the rate and equilibrium constants of the elementary steps.

The rate constant, k_4^a, was also determined by turnover experiments. Mixing AS1 with a stoichiometric amount or small excess of ATP gives dissociation, followed by reassociation as the ATP is exhausted. Since the ATP concentration changes during reassociation, the kinetic equations are nonlinear. k_4^a was determined by comparing experimental curves with the time dependence calculated by a computer solution of a simplified model. The rate of dissociation is proportional to ATP concentration and the conformation changes or hydrolysis steps of free S1 are fast compared to the rate of reassociation. The turnover was calculated from the model

$$AS1 + ATP \overset{k_1^a(ATP)}{\rightleftharpoons} A + S1\cdot ATP(=S1\cdot Pr)$$

$$S1\cdot Pr \overset{k_4^a(A)}{\rightleftharpoons} AS1 + Pr$$

The mechanism gives rise to four nonlinear differential equations for the time dependence of the concentrations of AS1, ATP, A, and S1·Pr. The first of these is $d(AS1)/dt = -k_1^a(ATP)(AS1) + k_4(A)(S1Pr)$. The equations were solved for AS1 and ATP by the Hemming predictor-corrector method (IBM scientific library subroutine MPCG). Since k_1^a is measured separately, the turnover curve depends on a single parameter k_4^a. Sets of curves for a range of values of k_4^a and various initial actin and ATP concentrations were drawn by a Calcomp 565 plotter for comparison with normalized experimental curves.

At moderate ionic strength (0.1 M KCl), the rate of acto-S1 ATPase becomes comparable with that of S1 ATPase ($k_4^a[A] \simeq k_7$ in eq 1). A second recombination pathway via steps 7 and 8 was included in the simulation under these conditions.

Similar computations were made for turnover experiments with acto-HMM but a simplifying assumption was made. The rate of dissociation is proportional to ATP concentration, but two molecules of ATP are probably required to produce dissociation (Sleep and Taylor, 1976). It was assumed that two molecules of substrate or product are carried around the cycle; i.e., the states are A·HMM, HMM·ATP_2, HM·Pr_2. Consequently, the kinetic equations have the same form as for acto-S1.

The rate constants were determined from measurements of turbidity or light scattering. The method assumes that the turbidity of acto-S1 (or acto-HMM) is proportional to concentration and that the contribution to the excess turbidity by an actin polymer, in which a fraction α of the sites is occupied by S1, is proportional to α. The first assumption is satisfied for acto-S1 and acto-HMM up to a concentration of 5 μM in actin residues. The second assumption requires the turbidity of acto-S1 complexes to be proportional to molecular weight. $T = KMC$, where $M = (m_a(1 - \alpha) + (m_a + m_s)\alpha)j$, m_a and m_s are the molecular weights of G actin and S1, j is the number of G actin units, and C is the molar concentration of F actin. Let $[c] = jC$ or Σjc_j if the F actin is polydisperse where [c] is the G actin concentration. The increase in turbidity of the complex relative to actin is $\Delta T = Km_s\alpha[c]$. The same equation holds for acto-HMM with m_s replaced by m_h, the molecular weight of an HMM head. Since excess turbidity is proportional to α, the rate constant is determined by substituting the time dependence of α into the turbidity equation. The assumption of a linear turbidity equation can be tested by turbidimetric titration of actin with S1 or HMM. The association constant is sufficiently large that α is proportional to S1 until the S1 concentration approaches the G actin concentration. In Figure 1, the increase in turbidity is plotted against initial S1 concentration for a range of actin concentrations from 0.4 to 5 μM. The equivalence point occurs at a mole ratio of 1 ± 0.1. The slope of the turbidity curve increases by almost a factor of two throughout the range. Similar results were obtained for 90° light scattering in the stop-flow apparatus and for the turbidity or scattering of acto-HMM. In the latter case, the curvature is slightly larger and the change in turbidity is approximately twice the value for acto-S1.

In an earlier study of the dissociation of actomyosin, a rough estimate of the error nonlinearity was made based on light-scattering theory (Finlayson et al., 1969). A more direct estimate can be made from the shape of the turbidity curve. For a dilute or ideal solution, scattering is proportional to $M^2P_{(\theta)}C$, where $P_{(\theta)}$ is a shape factor which takes account of the reduction in scattering from interval interference (Tanford, 1961). The ratio of the scattering of AHMM–AS1–A is 20:9:1, while the ratio of the squares of the corresponding molecular weights is 26:13.5:1. The increase in turbidity is determined primarily by the increase in molecular weight, while the interference factor decreases by about 30% for AHMM or AS1 compared to actin. The error in rate constants depends on the departure from linearity and an estimate of the maximum error should be obtained by calculating the rate constant obtained by taking the turbidity to be proportional to M^2, which slightly

overestimates the variation. Substitution for α using the rate equation for an association reaction, $\alpha = 1 - \exp(-\lambda t)$, gives a change in turbidity with time which shows a small deviation from a single exponential and the fitted rate constant is 30% too small in the case of S1. The experimental curves gave a reasonable fit to a single exponential, but recent measurements using a computer-fitting program have shown a small departure. In the case of dissociation, $\alpha = \exp(-\lambda t)$, the calculated curve gives a good fit to a single exponential, but the rate constant is 50% too large. Qualitatively, the errors are in the direction expected from the increase in slope of the turbidity curve with α. Half of the total turbidity change requires more than half reaction in association and less than half reaction in dissociation.

The turbidity method is useful because of its simplicity, but it should be substantiated by other techniques. Preliminary measurements of association and dissociation rates have been made using the quenching of the fluorescence of *N*-dansylaziridine-labeled actin by S1 and HMM. The rate constants are within 50% of the values obtained by turbidity and the discrepancy is in the direction predicted from nonlinearity of the turbidity signal (K. A. Johnson and E. W. Taylor, unpublished observations).

Results

Kinetic Measurements of Association of Actin with S1 and HMM. The change in turbidity for the reaction of S1 and HMM was measured over a range of concentrations with the S1 or HMM sites in excess over the equivalent G actin concentration. The increase in turbidity relative to actin fitted the equation $\Delta T = 1 - \exp(-\lambda t)$ as expected for a pseudo-first-order reaction. It is convenient to express the HMM concentration in terms of the number of binding sites (or heads), assumed to be 2/HMM molecule. The variation of λ with site concentration is shown in Figure 2. For both S1 and HMM, the rate was proportional to the concentration of sites in the range examined (up to 50 μM) and the rates were essentially equal. There was some variation among preparations but, within a range of ±25%, the rates were the same in all cases.

The evidence for S1 is consistent with a simple independent binding mechanism,

$$A + S1 \underset{k_{-s}}{\overset{k_s}{\rightleftharpoons}} AS1$$

and

$$\lambda = k_s[S1] + k_{-s}$$

The association of HMM satisfies a relation of the same form but the two heads of HMM are attached to two G actin residues based on the stoichiometry of binding determined by turbidimetric titration (Inoue et al., 1972; and this paper) and ultracentrifugation (Margossian and Lowey, 1973). The corresponding mechanism for HMM is

$$A + HMM \underset{k_{-h}}{\overset{k_h}{\rightleftharpoons}} A{\cdot}HMM$$

and

$$\lambda = k_h[HMM] + k_{-h}$$

but to be consistent the actin concentration should be expressed in units of G dimers. The quantity λ is determined by fitting the data to $1 - \exp(-\lambda t)$ for a range of HMM concentrations; consequently, the choice of the actin concentration unit does not affect λ or the numerical value of k_h. However, the form

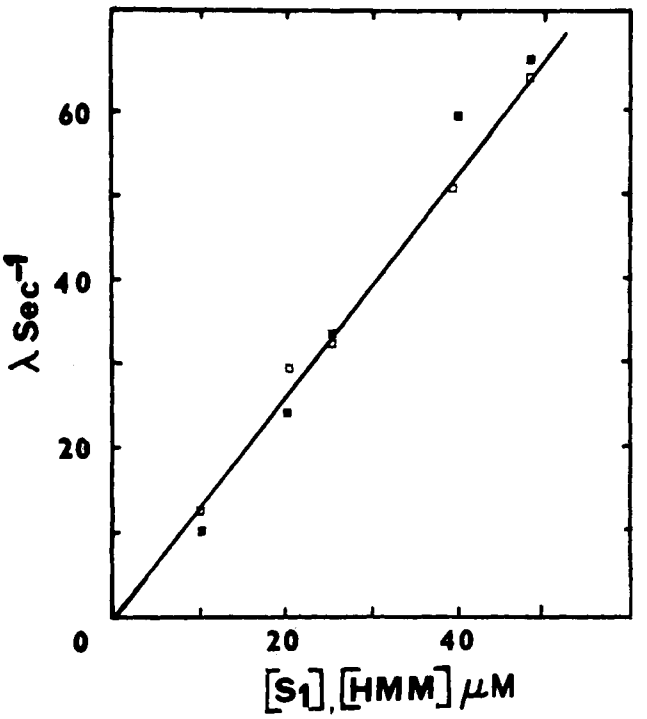


FIGURE 2: Rate of formation of the AS1 or AHMM complex as a function of the concentration of S1 or HMM sites. Rate constant calculated for a pseudo-first-order reaction with S1 or HMM sites in excess over actin, actin 5 μM, S1 (□), HMM (■). Concentration of HMM sites is twice the molar concentration of HMM. The conditions were otherwise identical to Figure 1.

of the equation includes an assumption regarding the steps in attachment of the two heads. In comparing the rate constants for S1 and HMM, it is preferable to express the rate per head, rather than per mole, to eliminate a difference of a factor of two which arises simply because HMM has two heads. The rate constant per head, k_h', is defined by $\lambda = k_h'[\text{heads}] = 2k_h'[HMM] = k_h[HMM]$. The rate constants for HMM in Table I should be multiplied by two to obtain k_h. It was found that the rate constant per head was approximately the same for HMM and S1; consequently, k_h is approximately $2k_s$.

The values of the rate constants are 1.4 and 1.3×10^6 M^{-1} s^{-1} for S1 and HMM association (100 mM KCl, 20 °C). The rate constant was strongly dependent on ionic strength. The values for S1 are 6×10^6 M^{-1} s^{-1} in 40 mM KCl and $\geq 2 \times 10^7$ M^{-1} s^{-1} in 10 mM KCl (Table I).

The intercept on the ordinate in Figure 2 gives k_{-s} or k_{-h}' and, since the intercept was essentially zero, the rate constant is <0.2 s^{-1}. The rate constant k_{-s} was estimated by measuring the rate of increase in turbidity for the reaction of a stoichiometric AS1 complex with excess HMM. The turbidity increased from the value for AS1 to that of AHMM with a rate constant of 0.02 s^{-1} independent of the HMM concentration. The result might be interpreted as the rate of spontaneous dissociation of S1, but the figure is probably an underestimate of k_{-s} because a single empty site may not be readily accessible to HMM. A better kinetic method has been developed by Dr. S. A. Marston based on the rate of exchange of S1 between unlabeled and fluorescent-labeled actin, which gives a value of 0.1 to 0.2 s^{-1} (S. A. Marston, personal communication). The "kinetic" value of the association constant is $1\text{-}2 \times 10^7$ M^{-1}. Equilibrium methods give values of $1\text{-}2 \times 10^7$ M^{-1} (Marston and Weber, 1975) (binding of radioactively labeled S1), 1.7×10^6 M^{-1} at 4 °C, and considerably higher at 20 °C

TABLE I: Kinetic Parameters of Actomyosin Reactions.[a]

Variable	Step 1, Apparent ATP Association $(M^{-1}\ s^{-1})10^{-6}$		Step 4, MPr Recombination $(M^{-1}\ s^{-1})10^{-4}$		Step 5, Specific ATPase $(M^{-1}\ s^{-1})10^{-4}$		Step 8, AM Association $(M^{-1}\ s^{-1})10^{-6}$	
	HMM	S1	HMM	S1	HMM	S1	HMM	S1
				Ionic Strength Dependence, 20 °C, pH 8				
100 mM KCl	1.2 ± 0.2	1.4 ± 0.2	0.8 ± 0.2	1.5 ± 0.2	1.0 ± 0.2	1.2 ± 0.2	1.4 ± 0.5	1.3 ± 0.5
40 mM KCl	1.4 ± 0.5	1.5 ± 0.3	12 ± 4	20 ± 5	15 ± 5	25 ± 5		6.0 ± 2
10 mM KCl	1.9 ± 0.5	3.0 ± 1.0	50 ± 20	60 ± 20	94 ± 10	92 ± 10		≳20
				pH Dependence, 20 °C, 40 mM KCl				
pH 7.0	2.5 ± 0.5	4.0 ± 1.0	15 ± 5	20 ± 1.0	20 ± 5	30 ± 5		
pH 8.0	1.4 ± 0.5	1.5 ± 0.3	12 ± 4	20 ± 5	15 ± 5	25 ± 5		
pH 9.5	1.1 ± 0.3	1.5 ± 0.3	2.0 ± 0.5	0.6 ± 0.2	2.9 ± 0.4	0.92		
				Temperature Dependence, 40 mM KCl, pH 8.0				
4 °C	0.7 ± 0.2	1.0 ± 0.3	0.15 ± 0.05	0.10 ± 0.05	0.23 ± 0.05	0.15 ± 0.05		
20 °C	1.4 ± 0.5	1.5 ± 0.3	12 ± 4	20 ± 5	15 ± 5	25 ± 5		
36 °C		3.0 ± 0.5		180 ± 50		200 ± 25		

[a] To facilitate comparison with the rate of recombination the actomyosin ATPase is expressed as specific actomyosin ATPase (AM rate − M) rate)/(actin concentration), 50 μM ATP. Rate constants or apparent rate constants given above refer to the scheme:

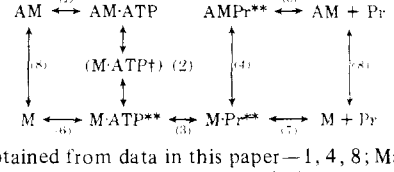
$$AM \underset{+ATP}{\overset{(1)}{\rightleftharpoons}} AM{\cdot}ATP \underset{-A}{\overset{(2)}{\rightleftharpoons}} M{\cdot}ATP \overset{(3)}{\rightleftharpoons} MPr \underset{+A}{\overset{(4)}{\rightleftharpoons}} AMPr \overset{(5)}{\longrightarrow} AM + Pr, A + M \overset{(8)}{\rightleftharpoons} AM$$

Conformation states are not specified. Rate constants for HMM reactions are expressed per site, assuming two identical sites per HMM. Buffers used: pH 7, 10 mM imidazole; pH 8, 10 mM Tris-Cl; pH 9.5, 10 mM sodium borate. For all conditions, the rate of dissociation (k_2) was greater than 500–1000 s^{-1}. Error limits refer to range for paired experiments or standard derivation for multiple determinations. The results for pH 8.0, 20 °C, 40 mM KCl are repeated in each block for ease of comparison.

TABLE II: Equilibrium Constants and Free-Energy Changes of Acto-S1 ATPase.

Step	K_{eq}	ΔG° (kcal)	ΔG_e° (kcal)
1	$10^6\ M^{-1}$	−8.1	−4.0
2	5×10^{-3}	+3.1	+3.1
3	10	−1.4	−1.4
4	$5 \times 10^2\ M^{-1}$	−3.6	−3.6
5	$2 \times 10^{-2}\ M^2$	+2.3	−8.5
6	$10^{11}\ M^{-1}$		
7	$5 \times 10^{-7}\ M^2$		
8	$2 \times 10^7\ M^{-1}$		
Total		−7.7	−14.4

[a] Values refer to 0.1 M KCl, 20 °C, pH 8. ΔG_e° calculated for ATP = 10^{-3} M, ADP = 10^{-5} M, P_i = 10^{-3} M, e.g., K_1(ATP) = (AM·ATP)/(AM), $K_1^e = K_1(ATP)$, $\Delta G_e^\circ = -RT \ln K^e$.



Constants obtained from data in this paper—1, 4, 8; Marston and Weber (1975)—K_8; Wolcott and Boyer (1974)—k_{-1}; Bagshaw and Trentham (1973)—K_3; Mannherz et al. (1974), Wolcott and Boyer (1974b)—K_6; K_2 calculated from K_1, K_6, K_8; K_7 calculated from K_6, K_3, and an equilibrium constant for ATP hydrolysis of 5×10^5 M; K_5 calculated from K_4, K_7, K_8.

(Highsmith et al., 1976; Highsmith, 1976) (fluorescence depolarization), and the order of $10^7\ M^{-1}$ to fit the shape of the turbidimetric titration, although this figure is subject to errors from nonlinearity of the turbidity relation. However, ultracentrifugation measurements give values which are one order of magnitude lower (Margossian and Lowey, 1973, 1976).

Dissociation of Actomyosin Complexes by ATP. The dissociation of S1 and HMM from actin is a pseudo-first-order reaction when ATP is present in excess as described previously for myosin (Finlayson et al., 1969) and HMM (Lymn and Taylor, 1971). The rate of dissociation of acto-HMM was proportional to the concentration of Mg-ATP to rates of at least 500 s^{-1} at 20 °C. Even at 0 °C, the rate was linear up to 500 s^{-1}. Identical results were observed for acto-S1. The dissociation of acto-S1 and acto-HMM by ATP can be compared at a particular ATP concentration or by the slope of the plot of rate vs. concentration of ATP, which defines the apparent second-order rate constant for ATP binding. The simplest mechanism is

$$AM + ATP \underset{}{\overset{k_1}{\rightleftharpoons}} AM{\cdot}ATP \overset{k_2}{\rightleftharpoons} \text{dissociation}$$

Values of the second-order rate constants are given in Table II. In 100 mM KCl, the rates of dissociation are equal for A·HMM and acto-S1 within an experimental error of ±20%. The reaction is relatively insensitive to ionic strength, increasing by about a factor of two between 100 and 10 mM, but at the lowest ionic strength the S1 dissociation may be slightly faster. Previous studies on actomyosin in 0.5 M KCl (Finlayson et al., 1969) gave a value of $2 \times 10^5\ M^{-1}\ s^{-1}$. The rate decreases by a factor of two as pH is raised from 7.0 to 9.5 and increases only threefold between 4 and 36 °C. Thus, the reaction is relatively insensitive to changes in pH, temperature, and ionic strength.

Recombination of the Intermediate Complex with Actin. The reaction of AS1 or AHMM with ATP when the ATP concentration is in small excess over the site concentration can be separated into three distinct phases. Figure 3 illustrates the turbidity changes at an ATP/site ratio of 2 for HMM. The turbidity shows an exponential decrease to a turbidity essentially equal to that produced by a large excess of ATP corresponding to nearly complete dissociation. The turbidity then remains constant for a period of time, which is proportional to the amount of ATP added. Finally, the turbidity increases to its original value. The regain in turbidity presumably occurs when the ATP is exhausted and the rate of the recombination step is a measure of the rate constant of association of the product–intermediate complex. It was first verified that re-

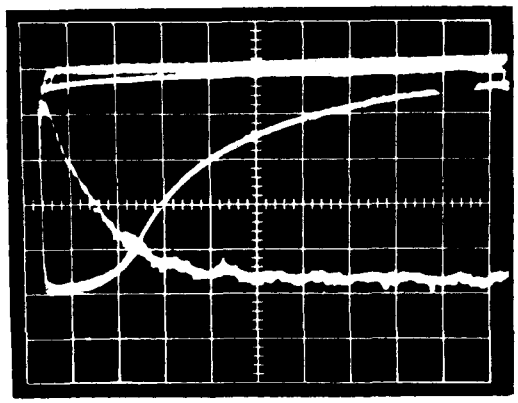


FIGURE 3: Dissociation of acto-HMM by ATP and recombination of HM·Pr_2 with actin. Syringes of the Aminco-Morrow stopped-flow spectrophotometer contained 10 μM acto·HMM (site concentration) and 20 μM ATP in 100 mM KCl, 10 mM Tris, 1.0 mM $MgCl_2$, pH 8.0, 20 °C. The time base for the exponentially decreasing signal is 50 ms/division and 5.0 s/division for the increasing signal. The vertical units which measure turbidity at 400 nm represent a change of 100 mV/division of a total signal of 8.0 V.

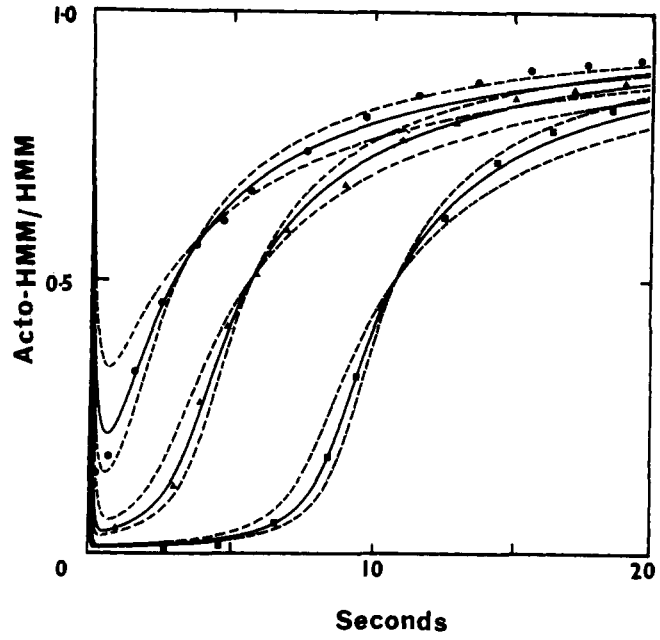


FIGURE 4: Recombination of HMM·Pr_2 with actin. Acto·HMM (10 μM actin, 10 μM HMM sites) was mixed with ATP in an experiment identical to Figure 3, except that the ATP concentration was 20 (●), 40 (▲), and 80 (■) μM, and KCl was 40 mM. The data were linearly scaled using the turbidity for actin and acto-HMM for complete dissociation and complete association, respectively. The solid curves were calculated as described in the text using $k_1^a = 10^6$ M^{-1} s^{-1} and $k_4^a = 1.6 \times 10^5$ M^{-1} s^{-1}. The dashed lines were calculated for $k_4^a = 1.2 \times 10^5$ M^{-1} s^{-1} and 2.0×10^5 M^{-1} s^{-1}.

combination occurs when ATP has been hydrolyzed by comparing the time period for regain of half the original turbidity with the rate of ATP hydrolysis measured in a pH stat. When the turbidity change is half complete, the amount of ATP remaining should be considerably less than 1 ATP/enzyme site; consequently, the recombination half-time should be approximately $[ATP]_0/v[AM]$, where v is the specific rate of ATP hydrolysis (moles/mole of actomyosin sites). The rate calculated from the half-time agreed with the direct measurement of the ATPase rate within 10–15% for a fourfold variation in actin concentration and the rate obtained from the recombination time was slightly slower, as might be expected. The measurement is useful, since it provides an internal check on the actomyosin ATPase activity of the preparation. The actomyosin ATPase rate is very sensitive to ionic strength and temperature and errors can be introduced in comparing the rate constant of recombination with the rate measured in a pH stat unless considerable care is taken to reproduce the experimental conditions in the two determinations.

Although the time course of the turbidity change is related to k_4^a, the apparent rate constant of the recombination step, the curve is distorted by the ATP remaining at the beginning of recombination, which can lead to a large error. The time course of the turbidity was, therefore, calculated from the solution of the kinetic equations for a simplified two-step model.

$$AM + ATP \overset{k_1^a(ATP)}{\rightleftharpoons} A\,MS, MPr \overset{k_4^a(A)}{\rightleftharpoons} AM + Pr$$

Measured values were used for k_1^a (Table I); thus, the time dependence depends on only one adjustable parameter, k_4^a. The approximations are discussed in a previous section (interpretation of rate measurement). Examples of normalized turbidity measurements and calculated curves are shown in Figure 4 for acto-HMM for three ATP concentrations. The solid curves correspond to the value of k_4^a obtained from the ATPase activity measured by the recombination half-time. This value gives the best fit to the turbidity data, since the dashed lines for each solid line were generated by changing k_4^a by ±25% and clearly give much poorer fits to the transient. Similar comparisons were made in which ATP was constant and the actin concentration was varied. A single parameter is sufficient to predict the time of maximum dissociation, the fractional dissociation, the slow increase phase, and the shape of the curve during the rapid recombination for a range of initial ATP and actin concentrations. We consider this to be a stringent test of the suitability of a two-step model and a relatively accurate measure of k_4^a.

Measurements were made over a range of temperature, pH, and ionic strength and the values were compared with the specific ATPase activity determined from the recombination half-time or pH stat measurements. The results are recorded in Table I. Reasonable agreement was obtained between k_4^a and the specific ATPase activity over a 1000-fold range in absolute values, although k_4^a was generally 20–40% low.

A second method was developed in order to measure the rate of recombination independently of a theoretical model. A stop-flow apparatus was constructed with two mixing chambers separated by a delay line. HMM or S1 was mixed with slightly less than a stoichiometric amount of ATP and the reaction was allowed to proceed for 1–2 s so that the MPr complex was formed and ATP was reduced to a small fraction of the MPr concentration. The sample was then mixed with actin in the second chamber. Recombination begins without a lag and with excess actin the reaction is pseudo-first-order. A comparison of rate of recombination and AS1 ATPase for various conditions is illustrated in Figure 5, which shows very good agreement between the two parameters. Within an error of ±30%, the rate constants measured by this method agreed with the values obtained by the fitting procedure. Both types of measurements were used to obtain the data listed in Table I.

Dependence on Temperature. An Arrhenius plot of the rate constant for the actin-activated S1 ATPase or the rate of the recombination step is linear and corresponds to an activation energy of approximately 150 kJ mol^{-1}. Similar high activation energies for acto-HMM ATPase have been reported by

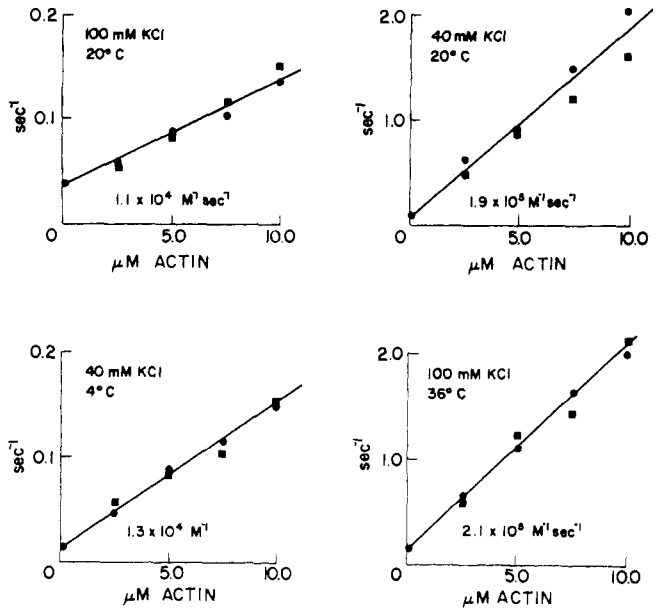


FIGURE 5: Dependence of the rate of AS1 ATPase and rate of recombination of S1·Pr with actin on concentration of actin. The rate constants for the recombination of S1·Pr + actin (■) were measured by the double-mixing procedure at the ionic strengths and temperatures shown. The rate constants for ATPase activity (●) were determined using a pH stat, as described under Materials and Methods.

Margossian and Lowey (1973) and by Barouch and Moos (1971). In contrast, the activation energies of the dissociation of acto-S1 by ATP and of the S1 ATPase are much smaller.

Conclusions

The primary purpose of these studies was to measure the apparent rate of recombination of the myosin-products state with actin and to compare this rate with the actomyosin ATPase for S1 and HMM. Measurements were made over a range of pH, temperature, and ionic strength which produce a 1000-fold variation in the specific actin activation. Over the entire range, the two rates were in reasonable agreement, although the rate of recombination was somewhat smaller, the discrepancy being less than a factor of two in all cases. The data recorded in Table I are average values which include the variation among preparations. It is necessary to consider the sources of error in the comparison. The actomyosin ATPase is very sensitive to temperature and ionic strength and errors are introduced if the conditions are not exactly reproduced in the two kinds of measurements. We have used an internal method of checking the ATPase activity by measuring the half-time for recombination.

A direct measurement of the rate constant for recombination cannot be made simply from the time course of the regain of turbidity. Recombination begins with ATP still present and the apparent rate has to be corrected. Since the kinetic equations are nonlinear, we have used a computer solution to obtain the best fit. The correction increases with increasing specific activation by actin, which may explain the large discrepancy found by Hozumi and Tawada (1974). In fact, by applying the computer-fitting method to their data and allowing that both HMM sites have ATPase activity, we obtained reasonable agreement between recombination rate and ATPase activity. To allow a direct measurement of the rate, a double-mixing technique was developed, which gave the same results as the corrected turnover experiments. Inoue et al. (1973) have also measured the rate of recombination by a double-mixing procedure for HMM in 50 mM KCl and pH 7.8. Their data shows a difference of a factor of two in the rates at 10 μM actin, and the rate of recombination is not proportional to actin concentration. It should be noted that we have compared the pseudo-first-order rate of recombination and the actomyosin ATPase activity per HMM site as a function of actin concentration. If the ATPase activity is expressed per HMM, there is a discrepancy of a factor of two.

Consideration of the errors introduced by nonlinearity of the turbidity relation and a preliminary comparison with a fluorescence method indicated that recombination rates should be 30–40% lower than the true values. The data listed in Table I are generally lower than ATPase rates by 10–40%. It is concluded that recombination of a product-intermediate state is the rate-limiting step in acto-S1 or acto-HMM ATPase for a wide range of experimental conditions, at least for actin concentrations up to 10 μM.

The rate constants for the association of actin with S1 or HMM were equal within experimental error. The values for the recombination of the product complexes fora range of conditions were larger for S1, although the difference was generally less than 50%. More accurate data are required but the rate constants expressed per head appear to be approximately equal for HMM and S1 reactions. Whether the rate constant for HMM should be equal to or larger than the value for S1 depends on the assumptions made in treating the attachment of the second head.

Comparison of the rate of the S1 association and S1·Pr association reactions as a function of ionic strength and temperature provides information on the mechanism. The rate constant for S1 association (k_8) has a small temperature dependence but decreases markedly with increasing ionic strength. A plot of $\log k_8 = a(\mu)^{1/2} + b$ is linear over the range from 10 to 120 mM in KCl, where μ is the ionic strength. At 20 °C, the rate constant extrapolated to zero ionic strength is 5×10^7 M^{-1} s^{-1}, which is approximately the value for a diffusion-controlled reaction (calculated from data in Benson (1960) and a diffusion constant of 2×10^{-7} cm^2 s^{-1}). The ionic-strength dependence shows that the rate constant is strongly influenced by a charge interaction which may raise the limit set by simple diffusion. In any case, it is clear that the protein–protein binding reaction is very fast and is comparable to enzyme–substrate reactions. The dependence on ionic strength satisfies the Debye–Huckel theory applied to the effect of ionic strength on the rate of reaction between two uniformly charged spheres. The slopes of the plots gave values for the product of the charges of −5.3 and −5.4 for the actin-S1 and actin-HMM reaction. Although the actual number is presumably not significant, since actin and S1 are neither uniformly charged nor spheres and have the same sign of the net charge at pH 8, the results suggest that there is a neutralization of charges at the binding site.

The apparent rate constant k_4^a for S1·Pr association has a similar dependence of rate on ionic strength. Apparently, the binding of the charged products does not influence the electrostatic contribution to the rate constant of association. The value of k_4^a is 30 to >100 times smaller than k_8, depending on ionic strength and temperature. In contrast to k_8, the change with temperature is very large and is the same as the temperature dependence of the actomyosin ATPase.

The minimum mechanism is

$$\text{S1·Pr} + \text{A} \underset{k_{-4}}{\overset{k_4}{\rightleftharpoons}} \text{AS1·Pr} \underset{k_{-5}}{\overset{k_5}{\rightleftharpoons}} \text{AS1} + \text{Pr}$$

If $k_{-4} \ll k_5$ then $k_4^a = k_4$. At 20 °C and 100 mM KCl, k_4^a

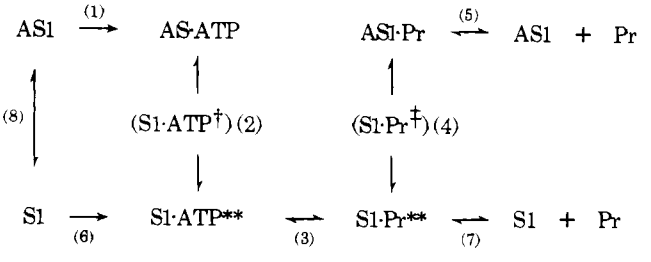
is only 10^4 M^{-1} s^{-1} and the value is even less at 4 °C. The values are sufficiently low to suggest that the process is not a simple binding reaction. One alternative is that the first step is a rapid equilibrium and the associated state is stabilized by a conformation change coupled to produce release at rate k_5. In this case, the rate of recombination is $K_4k_5[A]/(K_4[A] + 1) \simeq K_4k_5[A]$, since the measurements were made in a range in which the rate is proportional to actin concentration and $k_4^a = K_4k_5$. The V_{max} of acto-S1 ATPase is 10 s^{-1} but this figure may underestimate k_5, which could be 50 s^{-1} (Eisenberg and Kielley, 1972). Thus, K_4 is the order of 5×10^2 M^{-1}. This mechanism explains the large temperature dependence of k_4^a which is determined primarily by k_5. The large ionic-strength dependence is contributed by K_4. The equilibrium constant K_8 for the binding of S1 has a large ionic strength dependence (Highsmith, 1976) and it is reasonable to expect similar behavior for K_4.

The refractory state could also provide an explanation of the low value of k_4^a and it has recently been shown that the rate of recombination gives a hyperbolic dependence on actin concentration (Chock et al., 1976). Their simplest mechanism is

$$S1\cdot Pr \underset{k_{-r}}{\overset{k_r}{\rightleftharpoons}} S1\cdot Pr^{\ddagger} \overset{(4)}{\rightleftharpoons} AS1\cdot Pr^{\ddagger} \overset{(5)}{\rightleftharpoons} AS1 + Pr$$

where S1·Pr is the "refractory" state and $K_r < 1$ at low temperature and very low ionic strength. As shown by Chock et al., the rate of recombination is $k_r[A]/(K^a + [A])$, where $K^a = k_{-r}/k_4$ for the case of irreversible binding. At low [A], this reduces to $k_4^a = K_rk_4$. If the association step is a rapid equilibrium, the expression is more complex but at low [A] it reduces to $k_4^a = K_rK_4k_5$. Thus, including an extra state introduces the factor K_r into the expression for the apparent rate constant of recombination. Although an extra state may not be the only explanation of the turnover experiments at high actin concentrations, it is preferable to include the refractory state in interpreting the evidence. This explanation also leads to the conclusion that k_4 is larger than k_4^a and is still consistent with the interpretation of the association process as a rapid equilibrium.

The kinetic scheme for acto-S1 ATPase can be represented by the mechanism:



There is reasonable agreement on the S1 part of the pathway based on evidence from a number of laboratories. It is written in a simplified form here, since step 6 occurs in two stages (a rapid equilibrium for ATP binding and a conformation change) and step 7 occurs in two or three stages. Dissociation is a fast process following ATP binding and the rate has not been accurately measured for rabbit myosin. A maximum has been reached for myosins from slower muscles (Eccleston et al., 1976; Marston and Taylor, in preparation). The first dissociated state may be distinct from the states of the S1 pathway (S1·ATP†) (Sleep and Taylor, 1976) or identical with S1·ATP** (Chock et al., 1976). In either case, a transition monitored by fluorescence and ATP hydrolysis occurs after dissociation (step 3). Asterisks refer to states of enhanced fluorescence. The evidence presented here establishes that the pathway for recombination is from S1·Pr**. The results of Chock et al. may require a further state in the pathway (S1·Pr‡). Step 5 presumably occurs in two stages, since product release and a conformation change are required to complete the cycle. Specific labels are not assigned to possible conformations of the associated states through a lack of direct evidence. A similar scheme is satisfactory for acto-HMM ATPase, as the apparent rate constants expressed per head are very similar. It is not obvious that they should be the same and the question of interaction between heads remains to be answered.

Energetics of Actomyosin ATPase. A preliminary estimate of the equilibrium constants for the steps in the acto-S1 ATPase cycle can be given based on plausible assumptions. The values refer to 100 mM KCl and 20 °C. There are some difficulties in deciding which is the first dissociated state in the cycle and, for simplicity, S1·ATP** will be used. Step 1 probably occurs in two stages by analogy with S1 ATPase, but k_1^a/k_{-1} gives K_1 the overall equilibrium constant. k_{-1} is $\gtrsim 0.5$ s^{-1} which gives $K_1 \sim 10^6$ M^{-1} (Wolcott and Boyer, 1974a). The dissociation constant K_2 is equal to $K_6/(K_1K_8)$. For K_8, we take a value of 2×10^7 M^{-1} obtained from the kinetic measurements and the results of Marston and Weber (1975). K_6 is subject to error because of the lack of agreement between Mannherz et al. (1974) and Wolcott and Boyer (1974b) but a value of 10^{11} M^{-1} is probably correct to an order of magnitude. The assumption of a rapid equilibrium for S1·Pr** recombination gives a value of 5×10^2 M^{-1} for K_4 or K_rK_4 if the refractory state is included. $K_5 = K_4K_7/K_8$ and $K_7 \sim 5 \times 10^{-7}$ M^2 calculated from K_6, $K_3 = 10$ (Bagshaw and Trentham, 1973) and an equilibrium constant for ATP hydrolysis of 5×10^5 M. The value of K_5 is 2×10^{-2} M^2. The equilibrium constants are listed in Table II. The values obtained indirectly from relations among equilibrium constants are probably in error by at least an order of magnitude. However, the values serve to illustrate general features. The binding of substrate or products to acto-S1 is much weaker than to S1 by a factor of approximately 10^5. The binding of S1 substrate or product intermediates to actin is much weaker than the binding of S1, again by a factor of 10^4–10^5.

The binding of ADP or actin in the ternary complex AS1·ADP is weaker than the corresponding binary complexes (Beinfeld and Martonosi, 1975; Highsmith, 1976). AS1·ADP is, presuably, not equivalent to AS1·Pr and may stand in a similar relation as S1·ADP* to S1·Pr**, since the rate of ADP dissociation from AS1·ADP appears to be considerably larger than any reasonable estimate of k_5 (White, 1976). However, the binding studies support the general conclusion that dissociation constants of ternary complexes will be larger. Morales (1975) has discussed the relationship between the magnitudes of association constants in determining the energetics of the ATPase cycle and reached similar conclusions.

The standard free energy changes are listed in Table II but the values are somewhat misleading, since product dissociation has a positive free-energy change for 1 molar standard states of ATP, ADP, and P_i, and to illustrate the energy relations it is necessary to use values appropriate to muscle. Somewhat arbitrarily, concentrations of 10^{-3}, 10^{-5}, and 10^{-3} M for the three compounds were chosen to calculate the effective free energy values in Table II. The free-energy decrease for product release is about 8.5 kcal out of a total of 14.4 kcal, which is 60%. However, the actual free-energy change in the cyclic reaction is also affected by the ratio AMPr**/AM, which is

determined by all rate constants in the cycle (Taylor, 1973). Qualitatively, at a high ATP concentration the forward rates for the two states differ by a factor of 10–20; thus, the concentration ratio is about 10, which increases the free-energy change of the product release step to approximately 10 kcal. (Calculation of the free energy changes using plausible values of all rate constants verifies that the ratio AMPr*/AM is approximately 10.)

Models of muscle contraction generally assume that work is done by the attachment and rotation of a cross bridge (Huxley, 1969), and it is logical to identify this step with the formation of the AMPr complex and the product release step, which presumably includes a conformation change. We would expect that a property of a suitable model of the cycle in solution would be a large effective free-energy change for this step. A minimum of 50% of the total may be required, since muscle contraction efficiency can be 50%. A quantitative discussion of the relation between solution free-energy states and muscle work requires a detailed theory (Hill, 1974) and we are concerned here only with the point that the kinetic model appears to be energetically feasible.

In solution, the difference in effective free-energy levels can be increased by a concentration term which, in the formulation of Hill and Simmonds (1976a,b), corresponds to the difference between gross and basic free-energy levels. In the Hill-Simmonds model, this term does not contribute to the force. The distinction is mentioned only to avoid confusion since the actual numbers are crude estimates. It remains to be determined whether similar energy relations hold for acto-HMM and whether the mechanism suitable at moderate actin concentration is the dominant pathway in muscle.

Acknowledgments

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