Kinetic Studies on the Association and Dissociation of Myosin Subfragment 1 and Actin*

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(Received for publication, April 12, 1990)

The actomyosin ATPase cycle has been studied extensively as a basis for models of muscle contraction and motility (1). The mechanism is usually represented by a two-line "linear" scheme. A somewhat simplified form of the scheme is

\[
\begin{align*}
K_1 & \quad A^* + M \rightarrow AM, \\
K_2 & \quad A^* + M \cdot L \rightarrow AM \cdot L \\
K_3 & \quad AM \cdot L \rightarrow A + M \\
K_4 & \quad (A + M) \rightarrow A \cdot T \rightarrow AM \\
K_5 & \quad (A \cdot T) \rightarrow AM - D \cdot P \\
K_6 & \quad (A \cdot T) \rightarrow AM \cdot D \rightarrow AM \\
K_7 & \quad (A \cdot T) \rightarrow AM \cdot D \cdot P \\
K_8 & \quad (A \cdot T) \rightarrow AM \cdot D \cdot P \rightarrow AM \cdot D \cdot P - K_9 \\
K_9 & \quad AM \cdot D \cdot P - K_9 \rightarrow AM\cdot D\cdot P \\
K_{10} & \quad AM \cdot D \cdot P \rightarrow AM \cdot D \\
K_{11} & \quad AM \cdot D \rightarrow AM
\end{align*}
\]

\[
\begin{align*}
\text{SHEME I}
\end{align*}
\]

where \(M\) refers to a myosin head; \(A\) is actin; \(L\) is ligand; the asterisk refers to a high fluorescence state of actin; and \(K_3\) and \(K_4\) are association constants. \(K_1\) is reduced approximately 10-fold for \(M\cdot ADP\) or \(M\cdot pyrophosphate\) versus \(M\) alone. The rate constant of the isomerization step \((k_9)\) is 150--200 s\(^{-1}\) for \(A^* M\), \(A^* M\cdot ADP\), and \(A^* M\cdot pyrophosphate\ (20^\circ C)\). The interaction between the ligand and the actin binding sites reduces \(K_2\) from 2,000 for \(A^* M\) to 50--100 for \(A^* M\cdot ADP\) and to approximately unity for \(A^* M\cdot pyrophosphate\). The \(A^* M\cdot ADP\) state is equated with the \(A^* M\cdot ADP\) state of Sleep and Hutton (Sleep, J., A., and Hutton, R. L. (1986) Biochemistry 18, 1276–1283).

The occurrence of isomerization steps for actomyosin and actomyosin-ligand complexes affects the interpretation of the actin association constants in Scheme 1. \(K_4\) is the product of the two equilibrium constants for the formation of an initial complex and an isomerization whereas \(K_4\) refer only to the initial complex. It is an attractive hypothesis that the association constant for the initial step in myosin binding is equal to \(K_4\). Unfortunately \(K_4\) has been given different meanings depending on whether the authors are referring to the initial complex (2, 8, 9) or to the overall equilibrium (5, 12). These ambiguities can be avoided by using a three-line mechanism as discussed by Geeves et al. (7) in which an initial complex with actin is assigned to all of the myosin and myosin-ligand states, but the simpler formulation will be retained here.

The present study was undertaken to characterize the initial binding and the isomerization steps in the interaction of myosin and myosin-ligand complexes with actin. It is shown that the isomerization step has similar rate constants for myosin and myosin-ligand complexes, but the equilibrium constant is reduced by interaction with ligand. The initial complex formed between actin and M-D is identified with the AM-D state proposed by Sleep and Hutton (13). On this basis the steps in product release are reevaluated.

MATERIALS AND METHODS

Actin and myosin are prepared from rabbit psoas or leg muscles by methods described previously (4). Subfragment 1 prepared by chymotryptic digestion was separated into S1(Al) and S1(A2) by chromatography on DAE-Sephadex. The S1(Al) form was used in the experiments except as noted. Pyrene actin was prepared by labeling with \(N\)-(1-pyryl)iodoacetamide (14). F-actin in 100 m\(\text{M}\) KCl, 1 mm of MgCl\(_2\), 0.1 mm CaCl\(_2\), 0.2 mm MgATP, pH 7.5, was reacted with a 4:1 molar ratio of the label for 24 h at 4 °C in the dark. The label was dissolved in dimethyl sulfoxide just before use. Dichloroaceto-

*1 The abbreviations used are: S1(A1), S1(A2), myosin subfragment 1 isozyme with alkaline light chain 1 or 2; etheno-ADP 1-N'-ethenoadenosine diphosphate; AMP-PNP, adenosine 5'- (\(N\), \(N\)'-imino) triphosphate; PIPES, pipereazine-\(N\), \(N\)'-bis(2-ethanesulfonic acid).

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* This work was supported by Program Project Grant HL 20592 from the National Heart, Lung, and Blood Institute, National Institutes of Health, and by the Muscular Dystrophy Association of America. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
of the observed rate constant on concentrations was calculated from the values of \( k_{1-2} \) and \( k_2 \), and these values were adjusted if necessary to improve the fit to the experimental curve.

The apparent rate constant of dissociation, \( k_0 \), as determined by trapping free M with excess unlabeled actin, is obtained from the same equation by letting \( k_M \) approach zero. In this case, the square root can be expanded to give \( \lambda_2 = k_2 + k_3/k_2/k_3 + k_2 \). The ratio \( k_0/k_2 \) gives the overall equilibrium constant \( K_0/K_2 + 1 \).

The association of actin and myosin measured by light scattering fits the same equation as the fluorescence signal with the same rate constants \( \lambda_1 \) and \( \lambda_2 \) except that both states contribute to the increase in light scattering, \( \alpha_1 \) and \( \alpha_2 \) are negative, and the time course fits two exponential terms.

The reactions of SI-ligand complexes with actin, in which the ligands are ADP, AMP-PNP, and pyrophosphate were analyzed in terms of the scheme

\[
A^* + M \rightleftharpoons A^*M \rightleftharpoons AM \rightleftharpoons \text{free} M + \text{actin}
\]

The method of calculation is described under "Materials and Methods."
of the increase in light scattering was lost in the dead time of the apparatus (1.5 ms) at high S1 concentrations, but the remainder of the signal gave approximately the same rate constant as the fluorescence signal. The results are consistent with the scheme

\[ A^* + M \xrightarrow{(1)} AM^{(2)} \]

in which there is no measurable change in fluorescence in the formation of the initial complex as already described in Geeves and associates (10). The rate constant of the lag exceeds 1000 s\(^{-1}\) at high S1 concentrations, which would lead to the loss of most of the light scattering signal.

The data shown in Fig. 2 do not fit a hyperbolic dependence of the observed rate constant on S1 concentration. Therefore the concentration at half-maximum rate does not measure the association constant \( K_1 \). The maximum value of the apparent rate constant is \( k_2 + k_{-2} \), which is essentially \( k_2 \) since \( k_{-2} \) is very small. \( k_1 \) and \( k_{-1} \) were calculated by the procedure described under "Kinetic Equations." The values are 8 \( \times 10^5 \) M\(^{-1}\) s\(^{-1}\) and 50 s\(^{-1}\) for the experiment shown in Fig. 2. Therefore \( K_1 = k_1/k_{-1} = 1.6 \times 10^5 \) M\(^{-1}\). The calculated values of the individual rate constants are subject to relatively large errors. Average values of observed and calculated kinetic constants are summarized in Tables I and II.

The association reaction was also examined by varying the actin concentration at an actin to S1 ratio of at least 5:1. The relative signal amplitude is much smaller than for the experiments with S1 in excess, and a correction was made for bleaching of the fluorescence. The behavior was similar and gave a maximum rate of 200–250 s\(^{-1}\). Consequently, the observation that the reaction reaches a maximum rate is not explained by an interaction arising at high occupancy of the actin binding sites.

The apparent rate constant of dissociation of the actin S1 complex \( k_d \) was measured by mixing labeled actin-S1 with a 10–20-fold excess of unlabeled actin. The fluorescence signal fitted a single exponential term with a value of 0.025 ± 0.003 s\(^{-1}\) at very low ionic strength. The rate constant \( k_{-2} \) was calculated from the relation \( k_d = k_{-1} k_{-2}/(k_{-1} + k_2) \), which gave a value of approximately 0.1 s\(^{-1}\). Therefore, \( K_2 = 2,000 \).

The value of \( k_d \) increased with ionic strength, and at 0.1 M the value of 0.16 s\(^{-1}\) is in reasonable agreement with 0.1 s\(^{-1}\) given by Criddle et al. (10).

The value of \( k_d \) for unlabeled actin was obtained by the reciprocal experiment of displacement of S1 from the normal actin-S1 complex by an excess of pyrene labeled actin. The rate constant was measured for a range of ratios of labeled to unlabeled actin from 2 to 8, and the limiting value was obtained by plotting the rate constant versus the reciprocal ratio. The value was 30% smaller for normal actin than for labeled actin.

The modification of actin by labeling with a bulky pyrene group may be expected to affect the association with myosin. The value of \( k_a \) measured by light scattering was approximately equal for normal and labeled actin, but the maximum rate could not be determined accurately. Criddle et al. (10) estimated that the association constant was reduced by less than a factor of 2 by labeling of the actin.

The quantity \( k_d \) is equal to \( k_d k_a / (k_a + k_d) \). If \( k_d \) and \( k_a \) are unchanged but \( k_d \) is twice as large for normal actin, then \( k_d \) would increase by only 10–20%, which is consistent with the finding that \( k_a \) is similar for normal and labeled actin. Since \( k_d = k_d k_a / (k_a + k_d) \) a 2-fold increase in \( k_d \) would reduce the value of \( k_d \) by 30% to 50% for normal actin if \( k_d \) remained unchanged. Therefore the perturbation by labeling is consistent with at most a 2-fold reduction in \( k_d \) with very little change in the other rate constants.

The observed quantities \( k_a \) and \( k_d \) give the value of the association constant, \( k_d/k_a = K_2(K_2 + 1) = K_1 K_2 \) for \( K_2 \gg 1 \). The relationship is not sensitive to the number of steps in the mechanism. The values of the individual constants \( K_1 \) and \( K_2 \) are subject to the errors introduced in fitting individual rate constants, but the overall association constant of 2 \( \times 10^6 \) M\(^{-1}\) is obtained directly from the observed quantities.

The binding of S1(A2) to actin gave similar values for the kinetic constants. The association constant was reduced by a factor of 3 primarily by an increase in \( k_{-2} \). Cleavage of the S1 heavy chain by trypsin into the 25-, 50-, and 20-kDa segments reduced the association constant by 6–7-fold but had little or no effect on the isomerization step (\( k_d \)). The concentration dependence did not deviate sufficiently from a hyperbola to permit fitting of \( k_1 \) and \( k_{-1} \) separately. From the approximate relation \( k_a = K_1 K_2 \), the value of \( K_1 \) was reduced about 10-fold by trypsic digestion.

The association constant of S1(A1) to regulated actin in the presence of calcium ion was increased approximately 7-fold compared with nonregulated actin. Since the ionic strength was higher in the experiments with regulated actin 107 190 0.1 0.05 1.2 2.5 106

### Table I

| Conditions                   | Apparent second order rate constant (\(k_d\)) | Maximum rate constant (\(k_a\)) | Concentration for half-maximum rate | Apparent dissociation rate constant (\(k_d\)) | Equilibrium constant |
|------------------------------|---------------------------------------------|---------------------------------|------------------------------------|---------------------------------------------|----------------------|
| S1(A1)                      | (6 ± 2) \(10^7\)                           | 190 ± 10                        | 2.5 ± 1                            | 0.025 ± 0.003                              | 2.4 \(\times 10^6\) |
| S1(A2)                      | 4 \(\times 10^7\)                          | 175 ± 5                         | 2                                  | 0.05                                        | 8 \(\times 10^6\)    |
| S1(A1), regulated actin     | 7 \(\times 10^7\)                          | 170 ± 10                        | 1.2                                | 0.04                                        | 1.7 \(\times 10^6\) |
| Tryptic S1(A2)              | 1.2 \(\times 10^7\)                        | 150                              | 7.5                                | 0.1                                         | 1.2 \(\times 10^6\)  |
| S1(A1), 55 mM ionic strength | 4 \(\times 10^6\)                          | 25                               | 3.5                                | 0.05                                        | 25 \(\times 10^6\)   |
| S1(A1), 55 mM ionic strength | 2.5 \(\times 10^6\)                        | 225 ± 20                        | 7 ± 1                              | 0.1                                         | 2.5 \(\times 10^6\)  |
| S1(A1), 155 mM ionic strength | 0.9 \(\times 10^7\)                        | 350 ± 50                         | 30 ± 10                            | 0.27 ± 0.02                                | 4 \(\times 10^7\)    |
the increase in binding constant is closer to 10-fold at the same ionic strength in agreement with the findings of Geeves and Halsall (16). The rate constant of the isomerization step $k_2$ was not affected; the main difference was a 3-4-fold reduction in $k_5$.

A significant result of this series of experiments is that the rate constant $k_6$ of the isomerization step is essentially constant for S1(A1), S1(A2), tryptic-digested S1 and for the addition of regulatory proteins to the actin.

This rate constant has a large temperature dependence as might be expected for an isomerization step. At 6°C the rate constant $k_6$ was reduced to 25 s⁻¹. The concentration dependence gave an approximate fit to a hyperbola. The value of $K_l$ was reduced 2–3-fold. Although $k_6$ and $k_2$ could not be determined separately in this case the results are consistent with a small decrease in $k_6$ and increase in $k_2$. The large decrease in $k_6$ arises from the increase in the ratio of $K_l$ to $k_2$, since $k_6 = k_2/(1 + K_l/k_2)$.

Increasing the ionic strength decreased $k_2$ and the concentration dependence gave an increasingly better fit to a hyperbola. In 55 mM ionic strength $K_l$ was reduced by 10-fold. $k_2$ increased slightly with ionic strength. At ionic strengths larger than 100 mM, the fluorescence signal no longer gave a satisfactory fit to a single exponential term. At 155 mM ionic strength the rate constant of the main component extrapolated to a maximum rate of 350 ± 50 s⁻¹, but 50% of the amplitude fitted a second exponential term with maximum rate constant of 80 s⁻¹. A sequential mechanism is not consistent with both rate constants showing a dependence on S1 concentration. If S1 were a mixture of two different states at high ionic strength and the transition between these states had a smaller rate constant than the effective rate of binding to actin, the concentration dependence could be explained. However, this possibility was eliminated by mixing S1 at low ionic strength with actin at high ionic strength to give a final ionic strength of 155 mM. Both rate constants were observed, and the values were unchanged. The significance of a second rate process is not clear, and this aspect of the problem was not investigated further in the present work.

The main conclusions from investigating ionic strength effects are that the initial binding constant $K_l$ is markedly reduced by increasing ionic strength whereas the isomerization step shows only a small dependence on ionic strength. There was a small increase in $k_6$ and although $k_2$ increased, most of the change could be accounted for by the increase in $k_2$ rather than $k_5$.

### Table II

**Calculated rate constants for actin-S1 reaction**

| Protein                  | $k_1$ $M^{-1}$ s⁻¹ | $k_2$ $s^{-1}$ | $K_l$ $M^{-1}$ | $k_3$ $s^{-1}$ | $k_4$ $s^{-1}$ | $K_2$ $M^{-1}$ |
|--------------------------|---------------------|---------------|---------------|---------------|---------------|---------------|
| S1(A1)                   | (1.2 ± 0.2) × 10⁴   | 100 ± 50      | (1.2 ± 0.1) × 10⁶ | 195 ± 10      | 0.1 ± 0.03   | 1.9 × 10⁷     |
| S1(A2)                   | 6 × 10⁵             | 40            | 1.5 × 10⁷     | 175 ± 5       | 0.35         | 0.5 × 10⁷     |
| S1(A1), regulated actin  | 8 × 10⁵             | 20            | 4 × 10⁶       | 170 ± 10      | 0.035        | 5 × 10⁵       |
| S1(A1), 55 mM ionic strength | 1.2 × 10⁶            | 220 ± 20      | 0.15–0.2      | 2.2 × 10³     |
| S1(A1), 155 mM ionic strength | 3 × 10⁴               | 350 ± 50      | 0.3–0.4       | 1.2 × 10³     |
| S1(A1) 6°C               | 2.5 × 10⁵           | 25            | 0.05         | 0.5 × 10¹     |

### Reaction of S1-Ligand Complexes with Actin.

The reactions of S1-nucleotide and S1-pyrophosphate complexes with actin were investigated to determine the effect of the ligand on the rate and equilibrium constants of the mechanism established for the binding of S1 alone. The reaction has been studied previously using light scattering and fluorescent nucleotide analogues to determine the time course of association (2, 8, 17). The reaction was investigated using the change in pyrene fluorescence of labeled actin and compared with results obtained by light scattering. The kinetic behavior satisfied the same mechanism as the reaction with S1 alone. An extra step is added for the dissociation of the ligand.

$M \cdot L + A^* \rightarrow \frac{(1)}{K_1} A^* M \cdot L$, $\frac{(2)}{K_2} A^* M \cdot L$, $\frac{(3)}{K_3} A^* M + L$

where $K_1$ and $K_2$ are association constants, and $K_3$ is the first order rate constant of ligand dissociation.

The choice of ligand concentration in the S1 solution is a compromise. It has to be large enough to nearly saturate S1 because free S1 binds with a larger rate constant than S1-ligand. However, it must be small enough to dissociate from actin-S1 to prevent step 3 from affecting the apparent rate constant of the binding reaction. The ligand dissociation constants are 1 μM for S1, and 200–1000 μM for actin-S1. Ligand concentrations in the S1 solution before mixing were in the range of 10–40 μM in excess over the S1 concentration.

The reaction of S1-phosphorylate or S1-ADP with labeled actin gave a short lag in the fluorescence signal, and the remainder gave a good fit to a single exponential term. The light scattering signal was fitted approximately by one exponential term, but an increasing fraction of the total signal was lost in the dead time of the apparatus as the protein concentration was increased (12). Thus the behavior is essentially the same as for the binding of S1; the formation of an initial complex by a rapid reaction is followed by an isomerization detected by the pyrene label (Fig. 1). The dependence of the rate constant on the S1-ligand concentration gave a better fit to a hyperbola than for S1 alone. Data for two different protein preparations are shown in Fig. 3. The results for S1-ADP (circles) could be fitted by hyperbolae, giving maximum rate constants of 150–165 s⁻¹ and dissociation constants of 13–11 μM. The data points for S1-phosphorylate are not significantly different than those of S1-ADP and yield a maximum rate of 155 s⁻¹. The average values are given in Table III.

The reaction of S1-AMP-PNP with actin was complex.
Association-Dissociation of Actin-Myosin-Ligand Complexes

The reverse reaction, the dissociation of the actin-S1-ligand complex into actin and S1-ligand, was measured in order to determine the value of $k_{-2}$. In the case of pyrophosphate the rate of dissociation was measured as a function of pyrophosphate concentration. The rate constant measured by light scattering or pyrene fluorescence gave a hyperbolic dependence on pyrophosphate concentration, $K_p = 2 \times 10^5$ M$^{-1}$, and a maximum rate of 210 ± 20 s$^{-1}$ from fluorescence and 275 ± 40 s$^{-1}$ by light scattering (data not shown). Since the rate of association of S1-pyrophosphate with actin fitted a hyperbola, $k_{-2}$ is larger than $k_1$. An estimate of $k_{-2}$ was made by assuming that $k_1$ has approximately the same value as for S1 alone (10$^8$ M$^{-1}$ s$^{-1}$). From $K_p = 10^8$ M$^{-1}$, the value of $k_{-2}$ is approximately 500-1000 s$^{-1}$. Therefore the maximum rate of dissociation is determined primarily by $k_{-2}$. The maximum rate constants obtained by extrapolation for pyrophosphate-induced dissociation and for S1-pyrophosphate binding are approximately equal to $k_{-2}$ and $k_0$, respectively. However, these values are similar, and the observed rate constants also depend on $k_{1-2}$.

The rate of dissociation of S1-ADP from its complex with actin was determined by mixing labeled actin-S1-ADP with excess unlabeled actin. We have reported previously an apparent rate constant of dissociation of S1·ADP of 1·2 s$^{-1}$ (2). Labeled actin-S1 at a concentration of 5 µM and range of MgADP concentrations from 1 to 4 mM was mixed with 40 µM unlabeled actin. The increase in fluorescence fitted a single exponential term. The apparent rate constant increased with MgADP concentration as expected since the reaction scheme is

$$AM + D \rightarrow K_5 AM \cdot D \rightarrow k_{-2} A^* M \cdot D \rightarrow k_1 A^* M \cdot D + A_n A \cdot M \cdot D$$

where $A_n$ refers to normal (unlabeled) actin. The AM·D complex is essentially in rapid equilibrium with ADP, and the observed rate constant of the fluorescent signal is given by $[(ADP)/(K_5)/(1 + (ADP)/(K_5)) (k_{-2} + k_{-1})/(k_2 + k_{-2} + k_{-1})]$. The term in the first square bracket accounts for the decrease on MgADP concentration. The observed rate constant extrapolated to a value of 1.1 s$^{-1}$ with an apparent dissociation constant $1/K_5$ of 0.5-1 mM. As discussed above for S1-pyrophosphate, $k_{-1}$ is expected to be approximately 10$^5$ s$^{-1}$, which gives for $k_{-2}$ a value of 2 s$^{-1}$ at an ionic strength of 15 mM. At 120 mM, $k_{-2}$ is 4-5 s$^{-1}$, and $1/K_5$ is approximately 2 mM.

The apparent rate constant can also be determined from the maximum rate of dissociation of actin-S1 by MgADP. The method can be used only at high ionic strength and low protein concentration for which nearly complete dissociation can be obtained. The MgADP solution was preincubated with 0.5 µM S1 to hydrolyze any ATP present. At 100 mM ionic strength the increase in fluorescence gave a reasonable fit to a single exponential term although 10-15% of the amplitude fitted a larger rate constant. The maximum rate was 4-5 s$^{-1}$ at 3 µM actin. Measurements of the maximum rate over a range of concentrations. The maximum rate for the fastest process was 100 s$^{-1}$.

**TABLE III**

**Kinetic constants for S1-ligand reactions with actin**

| Ligand     | $k_1$ (µM$^{-1}$ s$^{-1}$) | Maximum rate (s$^{-1}$) | Concentration at half-maximum rate (µM) | $k_{-2}$ (s$^{-1}$) | $K_p$ (µM) |
|------------|---------------------------|-------------------------|-----------------------------------------|--------------------|-------------|
| Pyrophosphate | $1 \times 10^5$            | 100 ± 20                | 10 ± 2                                  | 220                | 250         | $2 \times 10^3$ |
| ADP        | $(1 \pm 0.4) \times 10^7$ | 11 ± 2                  | 1 ± 2                                   | 2 ± 3              | 2 ± 1       | $1 \times 10^6$ |
| AMP-PNP    | $5 \times 10^7$           | 100 ± 20*               | 3-5                                     | >50*               | >100        | $2 \times 10^9$ |

*The fluorescence signal fitted two exponential terms throughout the concentration range.

* Data from Ref. 2.
range of actin concentrations from 7.5 to 2.5 μM extrapolated to a value of 3-4 s⁻¹ at 0 protein concentration. Although the experiment is subject to error from extrapolation and to the problem of removing traces of ATP from the ADP solution the results confirm the value obtained by the displacement method.

The measurements of $k_3$ and $k_2$ yield an equilibrium constant of 50-100 for the isomerization step at very low ionic strength.

Comparison with Direct Measurements of ADP Dissociation from Actin-S1 — The kinetic scheme may appear to contradict previous studies since $k_3$ determines the rate of ADP dissociation. The rate constant of ADP dissociation from an equilibrium complex with actin-S1 as measured by the rate of dissociation of the complex by ATP is larger than 500 s⁻¹ (18) whereas $k_3$ is approximately 150 s⁻¹. However, $K_2$ is approximately 100, and at equilibrium the complex is essentially in the AM-D state, in terms of the simple kinetic scheme. Thus the rate constant measured for the equilibrium mixture is $k_3$ which is expected to be large. An estimate of the magnitude of $k_2$ was made by mixing S1-ADP with labeled actin plus a range of concentrations of ATP. The quenching of fluorescence in the formation of AM-D should be reversed at a rate determined by the dissociation of ADP and the binding of ATP to produce A⁺ plus M-T. The fluorescence signal $F$, normalized to the value for the complete association of S1 with actin is given by $F = [1/(r-1)] [\exp(-k_3t) - \exp(-K_2t)]$ in the limit of high ATP concentrations, where $r = k_3/K_2$, and $k_3$ is the effective (concentration-dependent) rate constant for the formation of AM-D. An example of a transient experiment is shown in Fig. 4. The actin solution contained 150 μM ATP. The increase in fluorescence is 8% of the value for full association. The falling phase of the signal has a rate constant of 66 s⁻¹, which is approximately equal to the value of 70 s⁻¹ obtained for the rate constant in the absence of ATP. Increasing the ATP concentration to 1 mM reduced the maximum change in fluorescence to 5-6% of the value for complete association. From the solution of the equation for the maximum value of $F$, $r$ is approximately 15, which corresponds with a value of $K_2$ greater than 1000 s⁻¹.

The rate constant for the dissociation of the fluorescent analogue etheno-ADP was measured previously in a similar type of experiment in which S1-etheno-ADP was reacted with excess actin containing MgATP (8). This experiment should measure $k_2$, but over the range of concentration from 15 to 70 μM actin the rate constant was approximately 400 s⁻¹ (Fig. 1 of Ref. 8).

There are a number of possible explanations of this discrepancy: a different rate constant for dissociation of etheno-ADP versus ADP; a different rate constant for normal versus pyrene-labeled actin; a difference in maximum rate for actin in excess versus S1-ADP in excess; a different step in the mechanism is measured by the quenching of actin fluorescence versus etheno-ADP fluorescence.

Eight data sets are necessary to test these possibilities. The results are summarized in Table IV. The errors are large for some combinations that necessarily produce small signals against a large background and a correction has to be made for bleaching of the fluorophor.

Rate constants obtained from the fluorescence change of pyrene actin reacted with S1-ADP and S1-etheno-ADP agreed within 10-20%. Etheno-ADP does not contribute to the change in fluorescence for excitation at 365 nm in the absence of acrylamide. However, with actin in excess the signal was fitted to one exponential plus a linear term to correct for bleaching. A deviation from a single exponential was also expected since measurements were made at an actin to S1 ratio of 4 in order to increase the relative change in fluorescence, and the reaction is not accurately first order. However, it is clear that ADP and etheno-ADP have essentially the same rate constant and that the rate constants for actin in excess are approximately the same as for S1 in excess.

The rate constant obtained from the quenching of etheno-ADP fluorescence was approximately twice as large for normal actin than for pyrene-labeled actin. The pyrene actin contributed a large background fluorescence which reduces the accuracy of the measurement. The experiment with normal actin in excess is in agreement with our previous results (8) except that the signal showed a small deviation from a single exponential term of about 10% of the total amplitude. In the previous study a deviation was found only at low temperatures.

**Table IV**

Comparison of rate constants of signals from etheno-ADP and pyrene actin

| Reaction                  | Fluorescence signal | Actin in excess | S1 in excess |
|---------------------------|--------------------|----------------|-------------|
|                          | Normal actin       | Normal actin   | Normal actin |
|                           | $s^{-1}$           | $s^{-1}$       | $s^{-1}$    |
| Etheno-ADP                | 130                | 275 ± 50       | 170         |
|                          | (110, 20)          | (350 ± 50, 50) | (200, 15)   |
| Pyrene actin              | 125 ± 20           | 160            |             |

**Fig. 4.** Dissociation of the actin-S1-ADP intermediate by ATP. Conditions: buffer as for Fig. 3; S1-ADP complex was mixed with actin plus ATP; final concentrations, 12.5 μM actin, 150 μM ATP, 5 μM S1, 20 μM ADP; quenching of fluorescence in the formation of AM-ADP and AM is plotted as an increase. The falling phase of the fluorescence curve is fitted by a rate constant of 66 s⁻¹. The maximum increase is approximately 8% of the value for formation of AM in the absence of ATP. Voltage amplification is 15 times larger than in Fig. 1.
ATP was present in the actin solution in the earlier experiments (8) to block rebinding of etheno-ADP and also to reduce changes in light scattering by dissociating the acto-S1 complex. This procedure gives a small improvement in the precision of the experiments, but the same values were obtained for the rate constants in the absence of ATP. It was omitted from this series of experiments in order to compare the two fluorescence signals under the same conditions.

This series of measurements is consistent with the simple scheme in which a single isomerization is detected by the change in actin or substrate fluorescence. A difficulty in the interpretation of these rate measurements is that a small contribution from a second exponential term that has a 10 times smaller rate constant is difficult to separate from an approximately linear term arising from bleaching of the fluorescence. The rate constant fitted to the main signal will be larger if the data are fitted by two exponentials rather than one exponential plus a linear term. Consequently the value of the rate constant \( k_5 \) may be overestimated, and a value of 300 s\(^{-1}\) will be assigned to the rate constant for normal actin.

**DISCUSSION**

Earlier studies (2, 17) led to the proposal that the minimum kinetic scheme must include one isomerization of actin-S1 or actin-S1-ligand complexes

\[
A^* + M \xrightarrow{K_1} A^* \cdot M \xrightarrow{K_2} A \cdot M, A^* + M \cdot L \xrightarrow{K_3} A^* \cdot M \cdot L \xrightarrow{K_4} AM \cdot L \xrightarrow{1/K_5} AM + L
\]

**Scheme 2**

where the asterisk refers to the high fluorescence state of actin, and \( K_1 \) and \( K_2 \) are association constants. The scheme accounts for most of the kinetic behavior. Although there are some deviations from the predictions of this model which will be considered below, the simple model permits all of the rate or equilibrium constants to be determined from the data. A comparison of the values for actin-S1 alone and for three ligands, ADP, pyrophosphate, and AMP-PNP, allows some generalizations to be made. For the binding of S1 alone K1 is large at low ionic strength (10\(^6\) M\(^{-1}\)), and \( k_1 \) is approximately 10\(^3\) M\(^{-1}\) s\(^{-1}\). The rate constant is in the range expected for a diffusion limited reaction of large proteins (19). However, electrostatic attractions in the binding site could increase \( k_1 \) relative to the value for simple diffusion, and some structural changes in the initial complex cannot be ruled out.

Step 2 is an isomerization of the complex, detected by a change in environment of the fluorophor on CyS\(^{354}\) of actin. \( k_7 \) decreased about 10-fold from 200 s\(^{-1}\) at 20 °C to 20 s\(^{-1}\) at 6 °C but showed only a small increase with ionic strength. The results indicate a conformational change of the actomyosin complex in the transition to the "rigor" state.

The binding of the ligands ADP, pyrophosphate, and AMP-PNP at the active site reduces the association constant \( K_1 \) by about 10-fold to 10\(^6\) M\(^{-1}\). A value of 2 × 10\(^5\) M\(^{-1}\) was obtained for normal actin and S1-ADP (2) versus 1.1 ± 0.2 × 10\(^5\) M\(^{-1}\) in this work; consequently, \( K_1 \) may be reduced slightly by labeling the actin. The rate constant for ATP or reaction products bound at the active site, the association constant is 2–3 × 10\(^4\) M (4). \( K_1 \) for S1 and for S1 products (20) is reduced by a similar factor by increasing the ionic strength; consequently the electrostatic contribution to the association constant is probably not affected. The results do not support the hypothesis that the initial complex formed with S1 has the same affinity as the "weakly" bound states. Rather there is a set of binding constants for S1 and its complexes with ATP, reaction products, and ADP which covers a range of about 50-fold.

Scheme 2 asserts that step 2 is a concerted change in structure which alters the environment of CyS\(^{354}\) of actin and of the base portion of etheno-ADP, two regions that are widely separated in the complex (21). It is difficult to test this interpretation by showing that the fluorescence signals from both labels give the same value of the rate constant because modification of the actin decreases the rate constant by up to a factor of 2. However within the limitations of the measurements both rate constants agree within 10–20% (Table IV). A second argument against sequential steps, such as \( A^* \cdot M \cdot D^* \rightarrow AM \cdot D^* \rightarrow AM \cdot D \), where asterisks refer to high fluorescence states of actin and nucleotide and \( e \) refers to etheno, is that the rate constants measured for the two fluorescence signal are nearly equal, and a sequential process would lead to an appreciable lag in the second transition. Particularly in the case of the pyrene signal the measurement is made with high accuracy (see Fig. 1), and the curve cannot be fitted by two sequential steps with similar rate constants. This is an important point, and further studies of this problem using a label on CyS\(^{354}\) of S1 (the –SH-1 sulfhydryl group) will be described elsewhere.

The rate constant of the transition \( k_5 \) is not greatly affected by ligands bound at the active site, which is some distance from the actin-S1 contact region. The interaction between the ligand site and the actin site is expressed primarily by the increase in \( k_2 \). The value of \( K_2 \) at very low ionic strength is about 2000 in the absence of ligand, 50–100 for ADP, and somewhat less than 1 for pyrophosphate and AMP-PNP. If the dissociation of actin-S1 by ATP is described by Scheme 2, the value of \( K_2 \) would have to be less than 10\(^{-2}\) to account for dissociation.

Geeves (22) has reported very different values for the rate constants of the reaction of the S1-ADP complex with pyrene-labeled actin \( (k_7 = 4 \text{ s}^{-1}, k_{d,2} = 0.4 \text{ s}^{-1}, k_{d,1} = 2 \text{ s}^{-1}, k_5 = 5 \times 10^4 \text{ M}^{-1} \text{s}^{-1} \text{ in } 60 \text{ mM KC1, 100 mM imidazole buffer} \) ). To compare our results with those of Geeves, a few experiments were conducted at higher ionic strengths. The constants \( k_2 \) and \( k_7 \) decreased, and \( k_5 \) increased with increasing ionic strength, but the change was less than a factor of 2–3-fold at an ionic strength of 65 mM compared with 15 mM. However, at higher ionic strengths the fluorescence transient for the association of S1-ADP with actin deviated from a single exponential term. A satisfactory fit was obtained to one exponential plus a linear term, and a better fit was given by two exponentials. In either case, \( k_7 \) is equal to or greater than 10\(^6\) M\(^{-1}\) s\(^{-1}\), and the maximum rate was 60–70 s\(^{-1}\) fitted to one exponential or 25 s\(^{-1}\) and 100 s\(^{-1}\) fitted to two exponentials. The disagreement between the stop flow results and the pressure relaxation results (24) is so large that the two experiments cannot be measuring the same process. In our earlier studies (2, 8) as well as in the present work, the effective rate of ADP dissociation has been shown to be large; consequently the slow step proposed by Geeves cannot be on the pathway described by Scheme 2. Although Scheme 2 is oversimplified and we cannot rule out a branch of the pathway which might account for the small change in association following the release of pressure, the very low value of \( k_7 \) and the linear dependence of the rate constant on protein concentration suggest that a very weakly bound intermediate state is responsible for the signal. For example, the value of \( k_7 \) is approximately equal to the value for the association of the M-D-P complex with actin (20).

The proposal (22) that \( A^* \cdot M \cdot D \) dissociates slowly \((k_{d,2}\) small) is not compatible with our data because of the much
larger value we obtained for \( k_a \). A further complication is that in (22) \( k_r \) was obtained from \( k_a \) using the approximation that the isomerization step is in equilibrium \((k_a = k_r/(1 + K_2) = k_r/k_a/(k_a + k_r))\). If \( k_r \) is equal or greater than \( k_a \) as appears to be the case, the complete equation \((k_a = k_r/k_a/(k_a + k_r + k_r))\) should be used which leads to significantly different results.

An important question is whether the isomerization of \( A^M \cdot D \) is a step in the hydrolysis cycle. Sleep and Hutton (13) introduced an additional intermediate in the cycle, AM' \(-\) D to account for the rate of ATP \( \leftrightarrow \) phosphate exchange and its lack of dependence on ADP concentration. AM' \(-\) D is present in appreciable amounts during the cycle, but at equilibrium a second AM' \(-\) D state is favored. An equilibrium constant of about 50 in favor of the AM' \(-\) D state is consistent with their data. It is evident that the properties of AM' \(-\) D correspond to the AM' \(-\) D state, which supports the proposal that it is a cycle intermediate. The product dissociation steps of the scheme of Sleep and Hutton are

\[
\begin{align*}
A^M \cdot D \cdot P & \rightarrow A^M' \cdot D \rightarrow AM' \cdot D + P \\
M \cdot D \cdot P & \rightarrow M' \cdot D \rightarrow M + P
\end{align*}
\]

where an asterisk is added to their notation to indicate the relation to this study and we have retained the numbering for the steps measured here. AM' \(-\) D and M' \(-\) D are collision complexes in equilibrium with free ADP. We have already discussed the evidence that step 2 is a concerted transition to AM' \(-\) D. The ADP reactions have to satisfy detailed balance; \( K_1/K_2 = 2 \times 10^6 \) \( \text{M}^{-1} \) and \( K_3/K_4 = 10^{-6} \) \( \text{M}^{-1} \) where \( K_6 \) and \( K_7 \) are defined in the direction of ADP binding. These values require \( K_3 \approx K_6 \approx 10^6 \text{M}^{-1} \). Although this value of \( K_7 \) agrees with equilibrium measurements (12) the approximate value from kinetics is \( 10^6 \text{M}^{-1} \). The discrepancy is large enough to raise the possibility that there is an additional step in the mechanism.

In a previous study the effective rate of product dissociation was calculated to be 80 \( \text{s}^{-1} \) although the value is model dependent (4). Since \( k_r \gg k_a \), the effective rate constant of product dissociation is \( k_rK_6/(k_a + k_r) \). A value of 300 \( \text{s}^{-1} \) for \( k_r \) gives roughly 100 \( \text{s}^{-1} \) for \( k_a \). The value of \( K_6/K_7 \) is 5–10. For these values of the constants \( A^M' \cdot D \) will be present in much larger amounts during the cycle than at equilibrium as required by Sleep and Hutton. The scheme still retains the property that the apparent dissociation constant of actin is larger than the \( K_a \) of actin activation of the ATPase (3, 4).

The dissociation constant of phosphate (\( K_a \)) is \( K_aK_a/K_a \) or roughly 500 mM (\( K_a \) is approximately 50 mM based on ATP \( \leftrightarrow \) P and P \( \leftrightarrow \) H\(_2\)O exchange measurements (13, 23, 24) and an equilibrium constant of three for the hydrolysis step for S1). A value of 1 mM can be calculated from data of Bowater and Sleep (25) for \( K_a = 100 \) and a hydrolysis equilibrium constant of three for actin-S1. The ATPase activity of cross-linked actin-S1 or myofibrils is barely inhibited by 100 mM of phosphate (data not shown) which is consistent with a value of \( K_a \) of at least 200 mM.

The scheme accounts for most of the kinetic and isotope exchange data, but it is not intended to be a complete description. Some kinetic studies of nucleotide association and dissociation cannot be explained by a single isomerization step (2, 8, 26), and a second isomerization might occur in the absence of nucleotide (9, 11). Although the scheme explains the increase in rate of nucleotide dissociation from actin-S1 (\( k_r \gg k_a \)) by the coupling of this step to an isomerization of the actin-S1 complex, it does not explicitly account for the increase in rate of phosphate dissociation (\( k_a \gg k_r \)). A logical extension of the mechanism is to include a second isomerization to drive phosphate dissociation. Also the scheme would not explain the inhibition of tension in muscle by phosphate in the 5 mM concentration range (27), which implies that some intermediate has a phosphate dissociation constant in this range. If step 4 is expanded to include an isomerization followed by rapid dissociation of phosphate this requirement would be met. For example if step 4 is replaced by \( A^M' \cdot D \cdot P \rightarrow A^M' \cdot D \cdot P \rightarrow A^M' \cdot D + P \) an equilibrium constant of 100 for the isomerization step would give a dissociation constant of 5 mM for phosphate. In an isometric fiber the A'M' \(-\) D state is expected to accumulate, and a phosphate dissociation constant of 3 mM was obtained by Bowater and Sleep (25).

Two actin-S1-ADP-P states have been invoked to explain steady-state properties (3), but the rate and equilibrium constants are different from those needed to explain phosphate binding to fibers.

The similarity in kinetic properties for the binding of myosin versus myosin-ligand complexes suggests that formation of an initial complex followed by one or more isomerization is an intrinsic property of the mechanism. The isomerization step or steps presumably corresponds to the change in structure of the complex which produces motion or tension. The isomerization of \( A^M' \cdot D \) is a global conformation change which is detected by labels placed in various regions of the complex. The isomerization of this state to give the rigor complex may contribute to tension. However, the A'M' \(-\) D state is probably itself a tension-generating state, and further evidence is needed to define the steps from the product intermediate state to A'M' \(-\) D.

Acknowledgment—I wish to thank Aldona Rukuiwa for expert technical assistance.

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