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Dynamic Interaction between Actin and Myosin Subfragment 1 in the Presence of ADP[†]

M. A. Geeves

Department of Biochemistry, School of Medical Sciences, University of Bristol, Bristol, BS8 1TD, U.K.

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ABSTRACT: The equilibrium and dynamics of the interaction between actin, myosin subfragment 1 (S1), and ADP have been investigated by using actin which has been covalently labeled at Cys-374 with a pyrene group. The results are consistent with actin binding to S1·ADP (M·D) in a two-step reaction, $\mathrm{A} + \mathrm{M{\cdot}D} \overset{K_1}{\rightleftharpoons} \mathrm{A{-}M{\cdot}D} \overset{K_2}{\rightleftharpoons} \mathrm{A{\cdot}M{\cdot}D}$, in which the pyrene fluorescence only monitors the second step. In this model, $K_1 = 2.3 \times 10^4\ \mathrm{M^{-1}}$ ($k_{+1} = 4.6 \times 10^4\ \mathrm{M^{-1}\ s^{-1}}$) and $K_2 = 10$ ($k_{+2} \leqslant 4\ \mathrm{s^{-1}}$); i.e., both steps are relatively slow compared to the maximum turnover of the ATPase reaction. ADP dissociates from both M·D and A–M·D at 2 $\mathrm{s^{-1}}$ and from A·M·D at $\geqslant 500\ \mathrm{s^{-1}}$; therefore, actin only accelerates the release of product from the A·M·D state. This model is consistent with the actomyosin ATPase model proposed by Geeves et al. [(1984) *J. Muscle Res. Cell Motil. 5*, 351]. The results suggest that A–M·D cannot break down at a rate $>4\ \mathrm{s^{-1}}$ by dissociation of ADP, by dissociation of actin, or by isomerizing to A·M·D. It is therefore unlikely to be significantly occupied in a rapidly contracting muscle, but it may have a role in a muscle contracting against a load where the ATPase rate is markedly inhibited. Under these conditions, this complex may have a role in maintaining tension with a low ATP turnover rate.

The cyclical interaction between actin and myosin driven by ATP hydrolysis forms the molecular basis of the crossbridge cycle which results in force generation in muscle. During each ATP hydrolysis cycle, the nature of the interaction between actin and myosin alternates between two defined states (Eisenberg & Greene, 1980; Geeves et al., 1984; Eisenberg & Hill, 1985). In the absence of nucleotide, actin is tightly bound to myosin ($K_d \simeq 0.1\ \mu\mathrm{M}$), and the rate at which actin dis-

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sociates from the complex is slow ($k_{\text{diss}} = 0.1\ \text{s}^{-1}$). In the presence of ATP, or when both ADP and P_i remain bound to myosin after ATP hydrolysis, actin is bound weakly ($K_d \simeq 1$ mM), and free actin is in rapid equilibrium with the myosin–nucleotide complex. It is during the return from this weakly attached state to the tightly bound state that force is believed to be generated, and this is thought to be associated with the release of the products P_i and ADP. The dynamics of the interaction between actin and the myosin product complexes are therefore central to the mechanism of force generation.

Several recent studies have explored the influence of the products of the ATPase reaction (ADP, P_i) on actin–myosin interactions in solution and on the crossbridge cycle in muscle. Cooke and Pate (1985) observed that the level of isometric tension in a skinned muscle fiber was elevated in the presence of ADP and that this was consistent with ADP inhibiting (competitively) the rate at which ATP bound to a tension-bearing state. White and his colleagues concluded from studies of a variety of muscles that there was a correlation between the temperature dependence of the rate at which ADP was released from its complex with actomyosin subfragment 1 (acto·S1)[1] and the maximum speed of shortening of the muscle, suggesting that ADP release could limit the shortening velocity (Siemankowski & White, 1984; Siemankowski et al., 1985). It had long been known that the ATPase reaction could not be readily reversed by the addition of ADP and P_i to either myosin S1 or acto·S1 but that when S1 was hydrolyzing ATP the addition of labeled P_i to the system resulted in the incorporation of label into protein-bound ATP (Boyer et al., 1973, 1975; Goody et al., 1977). Sleep and Hutton (1980) examined that acto·S1 ATPase using medium P_i exchange and showed that the exchange was much greater when ATP was being hydrolyzed than when ADP and P_i alone were incubated with acto·S1. They argued that the exchange was consistent with two acto·S1·ADP complexes being present in the steady state, one of which could not be readily formed by adding ADP to acto·S1. More recent work has used these results to support a model in which the force-generating event occurs between two acto·S1·ADP complexes (Hibberd et al., 1985). These studies suggest that acto·S1·ADP complexes may have an important role in the crossbridge cycle and a study of the dynamics of the interactions between actin, S1, and ADP is therefore of interest.

The interaction between actin and S1·ADP has been studied previously by both stopped-flow (Marston, 1982; Trybus & Taylor, 1980; Seimankowski & White, 1984) and pressure relaxation methods (Geeves & Gutfreund, 1982). These studies were consistent with a simple one-step binding reaction with $k_{+1} = 5 \times 10^4\ \text{M}^{-1}\ \text{s}^{-1}$ and $k_{-1} \simeq 0.1\ \text{s}^{-1}$ (0.1 M KCl, 20 °C, pH 7–8) although all of the above authors had some evidence of more complex reactions. In particular, the value of k_{+1} is too slow for a diffusion-controlled process and suggests that a rapid-equilibrium collision complex is also involved. More recently, the use of a fluorescent pyrene group covalently attached to Cys-374 of actin has provided a more sensitive measure of the degree of association between actin and S1, and this label has revealed the presence of additional acto·S1 and acto·S1·nucleotide complexes.

The pressure relaxation studies of Coates et al. (1985) revealed that S1 binds to actin in a two-step reaction and that pyrene fluorescence is quenched significantly only on the second step. The isomerization step is also the step which is perturbed by changes in hydrostatic pressure (Scheme Ia).

Scheme Ia

$$A + M \overset{K_1}{\rightleftharpoons} A\text{–}M \overset{K_2}{\rightleftharpoons} A\cdot M$$

More recent work has demonstrated that these same two states can be identified when nucleotide or nucleotide analogues are bound to myosin and that the value of K_2 is particularly sensitive to the nature of the occupancy of the nucleotide site on myosin (Geeves et al., 1986; Geeves & Jeffries, 1988). This two-step binding of actin to S1 was predicted in the model of the actomyosin ATPase reaction proposed by Geeves et al. (1984) in which a close link between the isomerization step and the force-generating event of the crossbridge cycle was discussed.

The results of a study of the interaction between pyrene-labeled actin and S1 in the presence of ADP are reported here, and two acto·S1·ADP complexes are identified. The relation between these two complexes and previously identified acto·S1·ADP complexes and the role of these complexes in the crossbridge cycle are discussed.

MATERIALS AND METHODS

Proteins. Myosin subfragment 1 was prepared by chymotryptic digestion of rabbit myosin, as described by Weeds and Taylor (1975). F-Actin was prepared by the method of Lehrer and Kerwar (1972). Pyrene-labeled actin was prepared as described by Criddle et al. (1985).

Pressure Relaxations. The pressure relaxation equipment has been fully described by Davis and Gutfreund (1976) and its use for inducing relaxations in acto·S1 and pyrene-labeled actin and S1 described by Coates et al. (1985). Pyrene fluorescence was excited at 365 nm and the emission monitored through a KV 393 glass filter. Light scattering was measured by monitoring the change in turbidity of the solution at 420 nm.

Stopped Flow. Rapid-mixing experiments were carried out on a Hi-Tech Scientific SF31 stopped-flow spectrophotometer (Salisbury England). Full details of the flow characteristics, optical arrangement, and the data handling are described in Geeves et al. (1986). Pyrene fluorescence was measured in the same way as for the pressure relaxation equipment; light scattering was measured at 90° to the 405-nm incident light. For the experiments where labeled actin was displaced from a complex with S1 and ADP by the addition of an excess of unlabeled actin, the addition of 1 μM cytochalasin D to both syringes improved the efficiency of mixing without changing the rate of the observed reaction.

Fluorescence Titrations. The fluorescence titrations were carried out on a Perkin-Elmer LS-5B fluorescence spectrophotometer. The fluorescence was excited at 365 nm and the emitted light monitored at 407 nm with a 2.5-nm bandwidth in each case.

RESULTS

Actin Binding to S1·ADP. The use of the pyrene fluorescence label on actin allows a simple titration to be performed in order to determine both the affinity of S1·ADP for actin and the effect of the pyrene label on the affinity. Such a titration is shown in Figure 1. The titration of actin which has been labeled to 98% with pyrenyliodoacetamide was fitted by using a nonlinear least-squares fitting routine to the quadratic equation:

$$[A]_0\alpha^2 - \alpha([A]_0 + [M] + K_d) + [M] = 0$$

where $[A]_0$ is the initial concentration of actin, [M] is the total

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[1] Abbreviations: S1, myosin subfragment 1; A, actin; M, myosin or its subfragments; N, nucleotide; D, adenosine diphosphate.

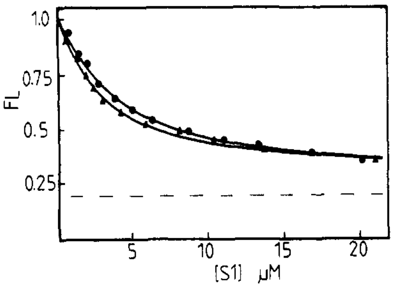


FIGURE 1: Fluorescence titration of pyrene-labeled actin with S1 in the presence of 2 mM ADP. S1 was added to a cuvette containing 2 μM actin labeled to >95% with pyrene at Cys-374 (▲) and labeled actin diluted 1:1 with unlabeled actin (●). The fitted line is the least-squares best fit as described in the text with K_d = 2.2 and 2.7 μM and F_{max} of 30.5% and 26% and F_0, respectively. The dashed line represents the fluorescence of 2 μM acto·S1 in the absence of ADP. Buffer conditions: 2 mM ADP, 60 mM KCl, 5 mM $MgCl_2$, and 0.1 M imidazole, pH 7.0, 20 °C.

concentration of added S1, K_d is the dissociation constant, and α is the fractional saturation of actin with S1. α is defined in terms of the fluorescence signal (F) as

$$\alpha = (F_0 - F)/(F_0 - F_\infty)$$

where F_0 and F_∞ are the fluorescence signals for zero and infinite S1 concentrations, respectively. The data were fitted by using the experimental values of $[A]_0$, F_0, F, and $[M]_0$ to yield the values of both K_d and F_∞. The best fit gave K_d = 2.2 μM and F_∞ = 30.5% of F_0. A repeat of the titration using labeled actin which has been diluted 1:1 with unlabeled actin is shown superimposed on the first titration and gives K_d = 2.7 μM, which is within experimental error of the previous measurement. The value of K_d obtained from these titrations is in good agreement with previous estimates using unlabeled actin by both equilibrium and kinetic techniques (Greene & Eisenberg, 1980; Marston, 1982; Geeves & Gutfreund, 1982), and the experiment demonstrates that the label has no significant effect upon the affinity of actin for S1·ADP. This is in agreement with previous work which has shown that the label does not affect the binding of actin to S1 in the absence of nucleotide (Criddle et al., 1985).

The fluorescence at infinite [S1·ADP] is 30.5% of F_0, which compares to 20% observed for S1 alone (shown as a dashed line in Figure 1). The extrapolation of the fluorescence signal to infinite [S1·ADP] may be subject to a substantial error, but the same result may be observed by adding 2 mM ADP to acto·S1 under conditions where no dissociation of the S1 takes place (the same buffer conditions but with 5 μM pyr·actin and 50 μM S1). Under these conditions, an increase in fluorescence from 20% F_0 to 28% is observed on adding ADP. This approach for studying acto·S1 nucleotide interactions has been exploited by Geeves and Jeffries (1988) where full experimental details are described. (An equivalent kinetic experiment is described in Figure 6iii.) Two possible explanations for the difference in the fluorescence of acto·S1 compared to acto·S1·ADP may be considered. In the first, the binding of ADP to S1 may perturb the environment of the pyrene label, producing the observed increase in fluorescence. The binding of ADP reduces the affinity of S1 for actin by more than a factor of 10, and it is reasonable therefore that the pyrene group could sense this change in affinity. The alternative explanation is that provided by the equivalent of Scheme I (Scheme Ib).

Scheme Ib

$$A + M\cdot D \underset{}{\overset{K_1^D}{\rightleftharpoons}} A\text{–}M\cdot D \underset{}{\overset{K_2^D}{\rightleftharpoons}} A\cdot M\cdot D$$

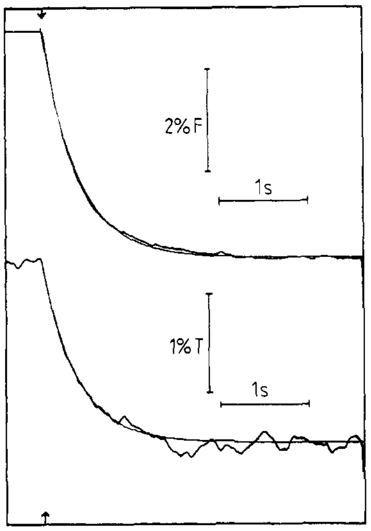


FIGURE 2: Pressure-induced relaxations of acto·S1·ADP. The effect of a rapid change in hydrostatic pressure from 10 MPa to atmospheric on a solution of 7.8 μM pyrene-labeled actin and 50.2 μM S1 in the presence of 2 mM ADP was monitored by (a) fluorescence and (b) transmission. A best-fit single exponential of 2.5 s^{-1} is superimposed in each case. The arrow indicates the time of pressure release. Other conditions as for Figure 1.

Coates et al. (1985) and Geeves et al. (1986) suggested that A–M and A–M·ATP had the same fluorescence as free actin but the value of K_2 changed from 200 in the absence of nucleotide to $\leq 10^{-2}$ in the presence of ATP. Thus, the change in fluorescence observed when ADP binds to acto·S1 could be accounted for if ADP reduces the occupancy of the low-fluorescence A·M·D state in favor of the high-fluorescence A–M·D state. An equilibrium constant (K_2^D) of $\simeq 10$ could be compatible with the results shown in Figure 1 (and the amplitude of fluorescence change in Figure 6iii).

Coates et al. (1985) were able to measure the equilibrium constant for the isomerization step in the absence of nucleotide from a study of the fluorescence amplitudes from pressure relaxation experiments. Geeves and Gutfreund (1982) had previously used this technique to study the kinetics of the interaction between actin and S1 in the presence of ADP using light scattering to monitor the reaction. The results from a pressure relaxation experiment in the presence of ADP using pyrene-labeled actin are shown in Figure 2. The results show that the relaxation when monitored by either pyrene fluorescence or light scattering is well fitted by a single exponential and both signals yield the same value of τ^{-1}. A plot of τ^{-1} against $[\bar{A}] + [\overline{S1\cdot D}]$ gives a straight line as shown in Figure 3A. These results are consistent with a single-step binding reaction where the gradient and intercept of Figure 3A define $k_+ = 4.6 \times 10^4\ M^{-1}\ s^{-1}$ and $k_- = 0.6\ s^{-1}$, respectively. However, consideration of the amplitudes of the relaxation shows that the model is too simple.

Figure 3B plots the observed amplitudes of the pressure-induced relaxations as a function of protein concentration and shows that fluorescence and light scattering have a markedly different concentration dependence. This result is incompatible with the one-step binding model, and the simplest alternative is the two-step model of Scheme Ib. The amplitudes predicted by any model more complex than a single-step reaction are highly dependent upon the details of the scheme and the precise values of the equilibrium constants. If the mechanism is that of Scheme Ib and it is like the reaction in the absence of nucleotide in that the only significant pressure perturbation occurs on step 2, and pyrene fluorescence monitors step 2 and light scattering step 1, then a reasonable fit to the data can

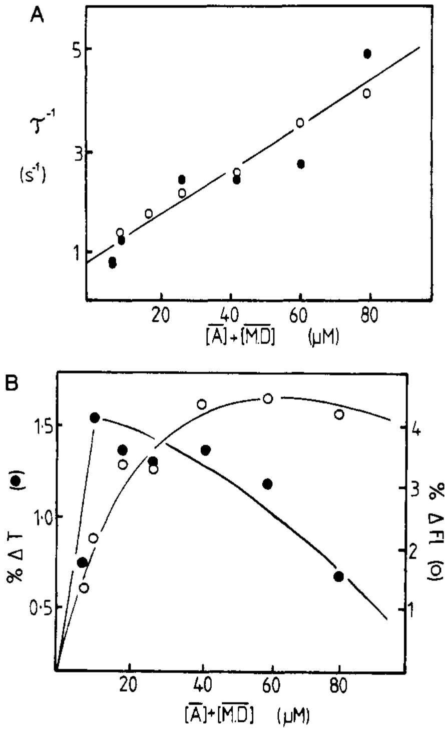


FIGURE 3: Dependence of the reciprocal relaxation time and the relaxation amplitude on protein concentration. Reciprocal relaxation time (A) and amplitude (B) for both fluorescence (○) and turbidity (●) signals plotted against the sum of free actin + free S1·ADP concentrations which were calculated from the dissociation constant obtained in Figure 1. The best-fit straight line to the fluorescence reciprocal relaxation times is superimposed and gives a gradient = 4.6×10^4 M^{-1} s^{-1} and intercept = 0.6 s^{-1}. The curves in (B) were fitted by eye.

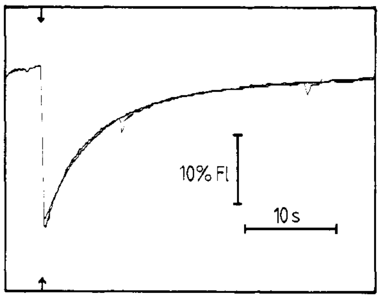


FIGURE 4: Displacement of pyrene-labeled actin from A·S1·ADP by the addition of excess unlabeled actin. 2 µM pyrene–actin and 1.95 µM S1 were mixed with 16 µM unlabeled actin in the stopped-flow fluorometer (concentrations after mixing). Both syringes contained 2 mM ADP and 4 µM cytochalasin D. The unlabeled actin syringe also contained 0.2 µM S1 to reduce the contaminating ATP in the ADP. The best-fit single exponential is superimposed with $k_{\rm obsd}$ = 0.18 s^{-1}. The arrows indicate the time at which flow stopped. Other conditions as for Figure 1.

be achieved. However, this cannot be taken as strong evidence to support this particular model, but the amplitude data of Figure 3B do exclude the single-step model and demonstrate that the pyrene fluorescence and the light-scattering signals are monitoring different events.

In the model of Scheme Ib, the gradient and intercepts of Figure 3A define the association rate constant ($k^{\rm D}_{+1}$) and the dissociation rate constant [$k^{\rm D}_{-1}/(1 + K^{\rm D}_2)$], respectively. The intercept is not well-defined by this plot, and a better estimate of $k^{\rm D}_{-1}/(1 + K^{\rm D}_2)$ was obtained by measuring it directly in a displacement experiment. Such an experiment is shown in Figure 4 where pyrene-labeled actin is displaced from A·M·D by the addition of a 5-fold excess of unlabeled actin. The fitted exponential gave a value of 0.18 s^{-1} for the observed rate, and increasing the concentration of unlabeled actin used in the experiment by a factor of 2 had no significant effect upon the observed rate. Therefore, $k^{\rm D}_{-1}/(1 + K^{\rm D}_2) = 0.18$ s^{-1}, and as the fluorescence titration suggested that $K^{\rm D}_2 \simeq 10$, then $k^{\rm D}_{-1} = 2$ s^{-1}. The value of the dissociation constant obtained in the titration (2.2 µM) is in reasonable agreement with the calculated dissociation constant, $k^{\rm D}_{-1}/k^{\rm D}_{+1}(1 + K^{\rm D}_2) = 3.9$ µM.

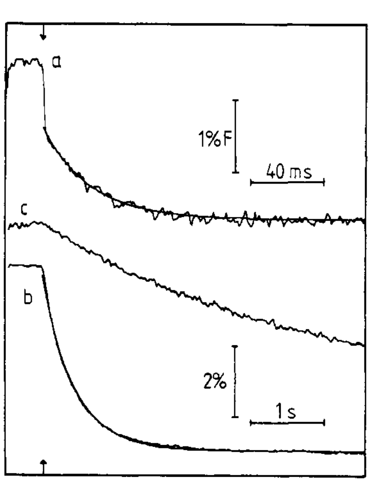


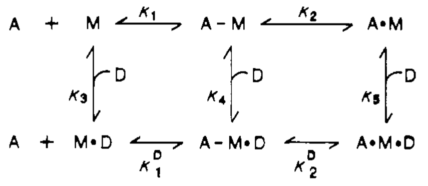
FIGURE 5: Effect of ADP on the pressure-induced relaxations of pyrene–acto·S1. Observed fluorescence changes on a 100-atm pressure jump on a solution of 7.9 µM pyrene–actin and 40.1 µM S1. The best-fit single exponentials are superimposed. Buffer conditions as for Figure 1. (a) No ADP, $k_{\rm obsd}$ = 38.8 s^{-1}; (b) 2 mM ADP, $k_{\rm obsd}$ = 2.3 s^{-1}; (c) the same trace as (b) but on a 5× faster sweep speed. The arrows indicate the time at which the pressure was released.

The experiment in Figure 2 shows only a single pressure-induced relaxation observed by fluorescence. In the absence of ADP, Coates et al. (1985) saw two relaxations: a fast relaxation which was only observed with fluorescence and was assigned to step 2 and a slow relaxation which had the same rate when monitored with either light scattering or fluorescence and was assigned to step 1. The total disappearance of the fast relaxation in the presence of ADP is demonstrated in Figure 5 where a relaxation on the same acto·S1 solution is shown before and after the addition of 2 mM ADP. Even on high gain, no evidence of a fast relaxation was observed in the presence of ADP, and the relaxation was well fitted by a single exponential. The two-step model can explain this result if, in the presence of ADP, the relaxation defined by $k^{\rm D}_{+2} + k^{\rm D}_{-2}$ (the fast relaxation in the absence of ADP) is indistinguishable from the relaxation defined by $k^{\rm D}_{+1}([{\rm A}] + [{\rm M{\cdot}D}]) + k^{\rm D}_{-1}(1 + K^{\rm D}_2)$ (the slow relaxation observed by both fluorescence and light scattering). The experiment was repeated with up to 110 µM free protein concentration, and in all cases, only a single relaxation was observed with a reciprocal relaxation time in the range 0.5–4 s^{-1}.

In order to be compatible with this result, the relaxation of step 2 must be unobservable over this wide concentration range. Step 2 may be unobservable either because the amplitude is very small compared to that of step 1 or because the rate $k^{\rm D}_{+2} + k^{\rm D}_{-2}$ is of the same order as $([{\rm A}] + [{\rm M{\cdot}D}])k^{\rm D}_{+1} + k^{\rm D}_{-1}$. This is possible if $k^{\rm D}_{+2} + k^{\rm D}_{-2} = 2$–$4$ s^{-1}, and at low protein concentrations (<20 µM), the amplitude is negligible as was observed for the reaction in the absence of nucleotide (Coates et al., 1985). Again, the model can fit the data, but the evidence is not compelling.

ADP Binding to Acto·S1. Further information on $k^{\rm D}_2 + k^{\rm D}_{-2}$ can be obtained by considering the rate of ADP binding to acto·S1. In order to discuss ADP binding to acto·S1, the combination of Schemes Ia and Ib must be considered as

Scheme II



shown in Scheme II. White (1977) measured the rate and equilibrium constants for ADP binding to acto·S1 by measuring the effect of ADP on the rate of the ATP-induced dissociation of acto·S1. He was able to show that ADP bound rapidly and reversibly to acto·S1 with an association constant of 6 × 10^3 M^{-1} and a dissociation rate constant of >500 s^{-1}. Scheme II would predict biphasic data in these experiments unless A–M·D and A·M·D are in rapid equilibrium or the rate of dissociation of ADP from the two states is comparable. However, data presented here suggest that neither of these cases applies. The slow phase of dissociation of ADP from A–M·D would have a small amplitude (10%) and, if the rate is slow, would be difficult to distinguish from light-scattering artefacts caused by aggregation. The experiments of White have been repeated using pyrene fluorescence to monitor the reaction. In this case, only a single process is predicted as the A–M·D state is a low-fluorescence state. Essentially results identical with those of White were obtained, and this then defines K_5 and k_{-5} in Scheme II.

The affinity of actin for S1·ADP is 20-fold lower than the affinity of actin for S1 alone (Geeves & Gutfreund, 1982; Greene & Eisenberg, 1980; Marston, 1982); therefore, the addition of ADP to 1 μM acto·S1 will result in significant dissociation of actin. The results of an experiment in which 100 μM ADP was mixed with 1 μM acto·S1 are shown in Figure 6i. The reaction was followed by both fluorescence and light scattering, and both show a single-exponential process with an observed rate of 0.7 s^{-1}. Increasing the concentration of ADP from 100 μM to 2 mM increased the amplitude of the observed reaction, as expected from equilibrium calculations, but had no significant effect on the observed rates (Figure 6ii). The rate of the observed reaction was independent of [ADP] for all ADP concentrations used between 20 μM and 2 mM. In the majority of experiments, particularly at higher ADP concentrations, some reassociation of actin with S1 appeared to take place over a period of about 1 min. This was believed to be due to the presence of contaminant ATP in the ADP as this effect could be eliminated by preincubating the ADP with Ap$_5$A and a catalytic amount of acto·S1. This treatment had little effect upon the observed rate of the dissociation. The absence of any dependence of the observed rate on the concentration of ADP is unusual, and the rate is rapid compared to the rate at which labeled actin is displaced from either A·M or A·M·D by an excess of unlabeled actin [0.22 s^{-1} for A·M (Criddle et al., 1985) and 0.18 s^{-1} for A·M·D (Figure 4)]. This suggests that the dissociation of actin can also occur by the other possible route of ADP binding to A–M to form A–M·D and then via k_{-1} to M·D.

This experiment was repeated at 50 μM S1 concentration, under which conditions little dissociation of actin will take place but the formation of A–M·D can be monitored by following the change in pyrene fluorescence. The light-scattering signal (Figure 6iii) shows no significant dissociation of acto·S1. The equivalent fluorescence signal shows a rate of 4 s^{-1} with an amplitude 10% of that expected for complete dissociation of acto·S1 as predicted by the fluorescence titration experiments in Figure 1. The rate of 4 s^{-1} can only be considered an upper limit for this reaction as it was difficult to be certain that the ADP was free of ATP. This result then allows the assignment of $k_{+2}^{D} + k_{-2}^{D} \leqslant 4$ s^{-1}, and as $K_2^D = 10$, then $k_{+2}^{D} \leqslant$ 3.7 s^{-1}. This then affords an explanation for why only one relaxation was observed in the pressure jump experiment. The previously defined value of k_{+1}^{D} predicts an observed rate for the relaxation of 0.6–5 s^{-1} over the concentration range 0–100 μM protein. The close correspondence between these rates and k_{+2}^{D} suggests that the rate measured in the relaxation experiment may be overestimated. This interpretation may also explain some of the deviations from single-exponential processes seen in the stopped-flow studies of actin binding to S1·ADP (Trybus & Taylor, 1980; Siemankowski & White, 1984).

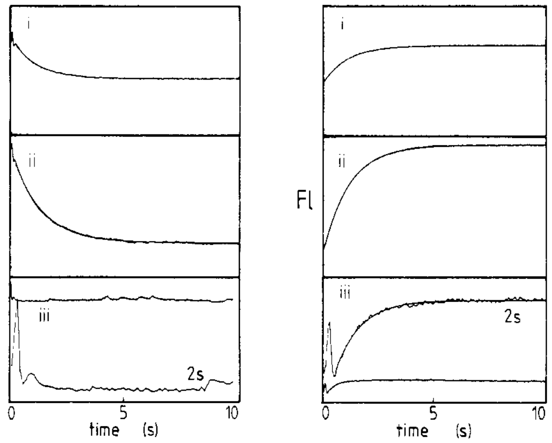


FIGURE 6: Rate of ADP binding to acto·S1. Observed changes in 90° light scattering and fluorescence on mixing ADP with acto·S1 under the following conditions (reaction chamber concentrations): (i) 1 μM actin, 1 μM S1 vs 100 μM ADP; (ii) 1 μM actin, 1 μM S1 vs 2 mM ADP; (iii) 1 μM actin, 50 μM S1 vs 2 mM ADP. In (iii), the trace is shown on the same scales as in the other two traces and in addition is shown amplified by a factor of 5 in both the time and signal scales. All other conditions as for Figure 1. The best-fit single exponential is shown superimposed in each case with the exception of the light scattering in (iii). The fitted rates and amplitudes, respectively, were as follows: for light scattering, (i) 0.93 s^{-1}, 0.17 V; (ii) 79 s^{-1}, 0.37 V; for fluorescence, (i) 0.91 s^{-1}, 0.45 V; (ii) 0.84 s^{-1}, 1.26 V; (iii) 3.9 s^{-1}, 0.23 V.

Only two steps of Scheme II remain to be defined. The binding of ADP to S1 has been studied many times and has been shown to be a relatively fast binding reaction with an association constant of 10^6 M^{-1} and a limiting dissociation rate constant of 2 s^{-1} (Trentham et al., 1976). This is known to be more complex than a single-step reaction, and the implications for this will be considered under discussion. Detailed balance then allows K_4 to be estimated as 5 × 10^5 M^{-1}. It should be remembered that equilibrium constants are rarely defined to better than a factor of 2, and so detailed balance estimates can only give approximate values. The pressure relaxation experiment in Figure 5 was repeated over a range of ADP concentrations, and the rate of the observed relaxation and the amplitude of the fast relaxation were plotted as a function of [ADP] (Figure 7). This shows that the effect of ADP on τ^{-1} saturates when ADP is stoichiometric with free S1. This is consistent with the free S1 binding ADP with an association constant (K_3) of 1 × 10^6 M^{-1} and with k_{+1}^{D} as 1% of k_{+1}. The amplitude of the fast relaxation decreases with increasing [ADP] but remains measurable until [ADP] is in excess of 200 μM. This is consistent with A–M, the species responsible for the fast phase, binding ADP with an association constant less than that for M. Modeling of the complete reaction of Scheme II suggests that K_4 is 20–50% of K_3 [(2–5 × 10^5 M^{-1}]. Modeling does not allow k_{-4} to be defined with

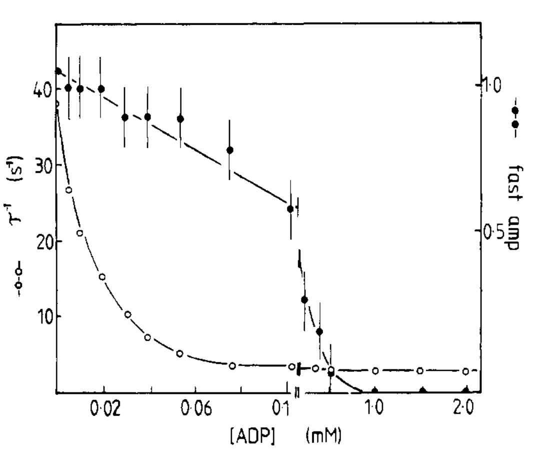


FIGURE 7: Dependence of the fast relaxation amplitude (●) and the slow reciprocal relaxation time (○), as defined in Figure 5, on ADP concentration.

Table I: Rate and Equilibrium Constants for Scheme II

step	K_i	k_{+i}	k_{-1}	ref
1	5×10^4 M^{-1}	1.4×10^6 M^{-1} s^{-1}	30	*a*
2	200	$\geqslant 2000$ s^{-1}	$\geqslant 15$ s^{-1}	*a*
1^D	2.3×10^4 M^{-1}	4.6×10^4 M^{-1} s^{-1}	2 s^{-1}	*b*
2^D	10	$\leqslant 3.7$ s^{-1}	0.37 s^{-1}	*b*
3	10^6 M^{-1}	2×10^6 M^{-1} s^{-1}	2 s^{-1}	*c*
4	$(2-5) \times 10^5$ M^{-1}	10^6 M^{-1} s^{-1}	2 s^{-1}	*b*
5	6×10^3 M^{-1}	$\geqslant 3 \times 10^6$ M^{-1} s^{-1}	$\geqslant 500$	*b*

[a] Geeves & Halsall (1986). [b] This work. [c] Trentham et al. (1976).

any precision, but the experiments of Figure 6i,ii limit k_{-4} to $\leqslant k_{-1}^{D}$ (2 s^{-1}).

DISCUSSION

The work presented here has allowed the rate and equilibrium constants of Scheme II to be defined, and these are summarized in Table I. However, this mechanism cannot be considered to be the complete description of the interaction between actin, ADP, and S1. There is a considerable body of evidence to suggest that the steps controlled by K_1, K_1^D, and K_3 are not single events. As shown, these steps are bimolecular association reactions, yet in no case has the reaction been shown to have the properties expected for a diffusion-controlled reaction. In addition, there is evidence from ^{31}P NMR (Shriver & Sykes, 1981) and from the use of both protein fluorescence and fluorescent analogues of ADP that two M·ADP states exist in equilibrium with each other (Trybus & Taylor, 1982; Rosenfeld & Taylor, 1984; Smith & White, 1985). The implications of these additional features will be considered later, but it will be suggested that such considerations do not fundamentally alter the principal features of the mechanism of Scheme II.

Several features of Scheme II are worth emphasizing: The equilibrium constants of the protein isomerization steps (K_2 and K_2^D) have a similar dependence on ionic strength and the presence of ethylene glycol (Geeves & Jeffries, 1988). This suggests that a similar structural change is taking place even though the rate of the structural change ($\geqslant 2000$ s^{-1} compared to $\leqslant 4$ s^{-1}) and the equilibrium position ($K_2 = 200$ compared to 10) are markedly influenced by the presence of ADP. ATP influences this isomerization in a very different way; i.e., it remains fast (3000 s^{-1}), but the A·M·T state is only transiently occupied ($K_{eq} \leqslant 0.01$) (Geeves et al., 1986).

The rate of release of ADP from S1 is not accelerated by actin binding alone. This only occurs when the A·M·D state is attained: i.e., the binding of actin to form the A–M·D state does not weaken the binding of nucleotide significantly even though the presence of ADP does reduce the rate (but not K_1) of actin binding. This observation is consistent with the model of Geeves et al. (1984) which proposed that the A–M·N to A·M·N transition (N = nucleotide) is linked to the force-generating event in muscle. Thus, product release is not accelerated until after this energy-transducing transition has occurred.

Slow rates appear to be a general feature of the actin/S1 interaction in the presence of ADP. All the steps in the presence of ADP are slow apart from the rate of dissociation of ADP from A·M·D (step 5). Actin in the A–M·D state, although relatively weakly bound, is not in rapid equilibrium with free actin as is actin in the equivalent A–M and A–M·T states. In fact, the A–M·D state is limited to a rate of breakdown of $\leqslant 4$ s^{-1} whether ADP dissociates, actin dissociates, or the complex isomerizes to A·M·D.

It is relevant at this point to consider the two acto·S1·ADP states identified here in relation to ternary complexes identified by other studies. Siemankowski et al. (1985) proposed that the loss of ADP from the predominant aco·S1·ADP state formed on equilibrating the three components together (A·M·D in Scheme II) could be involved in limiting the overall crossbridge cycling rate in a rapidly contracting muscle. The evidence for this was a high correlation between the temperature dependence of the in vitro ADP dissociation rate constant (k_{-5} of Scheme II) and the maximum speed of shortening for a wide variety of muscle types. This contrasts with the view proposed by Sleep and Hutton (1980), who pointed out that this A·M·D complex may not be on the main ATPase pathway and therefore may not be occupied significantly in a muscle contracting under physiological conditions. These workers identified a further acto·S1·ADP complex which can bind phosphate but which is not readily formed on equilibrating actin, S1, and ADP, although it is present during ATPase turnover. The data require that this additional acto·S1·ADP state is present at $\simeq 50$ times the concentration of A·M·D during ATP turnover, that the equilibrium between the two is 50 in favor of A·M·D, or a combination of the two. The data presented here suggest a value of 10 for K_2, but this rises to $\geqslant 20$ at the lower ionic strength used by Sleep and Hutton (Geeves & Jeffries, 1988), which suggests that A–M·D is compatible with their second acto·S1·ADP state. If this is correct, then A–M·D must bind phosphate more tightly than A·M·D, and this is currently under investigation.

An interesting feature of the A–M·D state is that if this state is formed by the loss of phosphate from a A–M·D·P$_i$ state during ATP turnover, then the further breakdown of this state is limited to $\leqslant 4$ s^{-1}, which is significantly less than the turnover rate of the ATPase (20 s^{-1}). Accumulation of this state would therefore result in inhibition of the ATPase reaction. This state is therefore unlikely to be significantly occupied during rapid ATP turnover in solution or in a rapidly shortening muscle. However, under conditions where the ATPase rate is considerably reduced, this state could become significantly occupied. For example, in an isometric contraction where the muscle is prevented from shortening, the ATPase rate is considerably inhibited (1–3 s^{-1}), and the A–M·D could as a result become significantly occupied. Indeed, if the transition from A–M·N states to A·M·N states is associated with the force-generating event, as proposed by Geeves et al. (1984), then the presence of strain in an isometric muscle would, by Le Chatelier's principle, result in a higher occupancy of the A–M·N states.

In this view of the overall reaction in a rapidly shortening muscle, the predominant route would be A–M·D·P$_i$ →

A·M·D·P$_i$ → A·M·D → A·M (where A–M·D·P$_i$ and A·M·D·P$_i$ are the equivalent acto·S1 states with both ADP and P$_i$ bound), leaving the A–M·D state unoccupied. In a muscle developing tension, a contribution would come from the route A–M·D·P$_i$ → A–M·D → A·M·D → A·M. This possibility affords an explanation for the slow rate of association and dissociation of actin with M·D because even though the A–M·D state cannot contribute to tension generation actin remains attached and therefore will not allow lengthening of the fiber which would result in tension decline. In this view, the A–M·D state is analogous to the latch state of molluscan muscle which can maintain tension for long periods without utilizing ATP. In this case, the lifetime of the crossbridge is very much shorter (0.2–1 s) but such an attached crossbridge could help to maintain tension with an ATPase rate lower than that in a shortening muscle. Such a crossbridge state would also initially inhibit shortening if the load on the fiber was suddently reduced.

The view of the role of acto·S1·ADP states outlined here is compatible with the model of Siemankowski et al., where in a shortening muscle A·M·D is a significant state, and with the presence of two acto·S1·ADP complexes as proposed by Sleep and Hutton.

Any attempt to correlate the results presented here with muscle fibers directly faces several major difficulties. Many of these have been discussed in a review by Hibberd and Trentham (1986), particularly the strain dependence of rate constants. In addition, information of the effects of the presence of troponin and tropomyosin, in the presence of calcium, on individual rate constants is not well documented although several studies have addressed this point (Trybus & Taylor, 1980; Geeves & Halsall, 1986; Rosenfeld & Taylor, 1987). Similarly, the effect of the second head in heavy meromyosin has not been established. When two acto·S1 states are both significantly populated, as is the case for the complex with ADP, then small changes in the binding energies caused by interactions between the heads or by the presence of the control proteins could have quite major effects upon the relative population of the two states. Therefore, caution has to be used in extrapolating these studies on acto·S1 to muscle in anything other than the most general terms.

Diffusion-Controlled Reaction. The bimolecular association reactions controlled by K_1, K_1^D, and K_3 are expected to have the properties of diffusion-controlled reactions, yet in no case has this been demonstrated. Bagshaw et al. (1974) demonstrated that the association of ADP with S1 (K_3) was at least a two-step reaction; a bimolecular association step followed by an isomerization, and this has been confirmed by subsequent studies.

The rate constant k_{+1} is almost fast enough for step 1 to be a diffusion-controlled reaction at 20 °C, but its activation energy is far too high to be a simple bimolecular association reaction. For this reason, Coates et al. (1985) suggested that the simplest interpretation was that step 1 is itself a two-step reaction; a collision complex precedes the formation of the attached state which is then followed by the isomerization shown in Scheme I.

$$\mathrm{A} + \mathrm{M} \overset{K_0}{\rightleftharpoons} (\mathrm{A}{\sim}\mathrm{M}) \overset{K_1}{\rightleftharpoons} \mathrm{A{-}M} \overset{K_2}{\rightleftharpoons} \mathrm{A{\cdot}M}$$

The association of actin with M·D (controlled by k_{+1}) is far too slow and has too strong a temperature dependence to be a diffusion-controlled reaction (Geeves & Gutfreund, 1982; Marston, 1982; Seimankowski et al., 1985), and the simplest explanation is that, as for the reaction with S1 alone, a collision complex is formed before the formation of the A–M·D state.

The presence of these additional collision complexes in the reaction does not alter the interpretation of Scheme II as the collision complexes are rapid equilibrium states and they are not significantly occupied under most experimental conditions. The exception is the bimolecular collision complex formed between ADP and acto·S1 which cannot be distinguished from A·M·D which is significantly occupied. The presence of the collision complexes is important if the process of association is to be understood in terms of initial complex formation followed by specific bond formation at the recognition sites of the protein–protein interface.

Two S1·ADP Complexes. Several studies of ADP binding to S1 (and to acto·S1) have suggested the presence of at least two S1·ADP states in addition to any collision complex. The discussion so far has assumed the presence of only a single population of S1·ADP states. If there were additional S1·ADP complexes, then they would appear as a single population if they were interconverting at a rate ≥10 s^{-1} as all the observed reactions are occurring at a rate much below 10 s^{-1}. The NMR and protein fluorescence studies suggest that the two S1·ADP complexes are interconverting at 110 s^{-1} at 20 °C and at 15 s^{-1} at 3 °C (Shriver & Sykes, 1981; Trybus & Taylor, 1982). However, the presence of such an equilibrium of S1·ADP states could produce an apparent rate of association with actin much lower than the true association rate. If there were two S1·ADP states (M·D and M′·D) and the predominant state was unable to bind actin significantly, then this would have to isomerize before binding to actin could take place (Scheme III).

Scheme III

$$\mathrm{M'{\cdot}D} \overset{K_0^D}{\rightleftharpoons} \mathrm{M{\cdot}D} + \mathrm{A} \overset{K_1^D}{\rightleftharpoons} \mathrm{A{-}M{\cdot}D} \overset{K_2^D}{\rightleftharpoons} \mathrm{A{\cdot}M{\cdot}D}$$

This scheme predicts that the apparent affinity of actin for S1·D (K_{app}) is defined by

$$K_{\mathrm{app}} = K_1^D(1 + K_2^D)/(1 + 1/K_0^D)$$

where $K_0^D = [\mathrm{M'{\cdot}D}]/[\mathrm{M{\cdot}D}] > 1$. The assignment of $K_2 = 10$ is unaffected by this change, and therefore $K_1^D/(1 + 1/K_0^D) = 2.3 \times 10^4\ \mathrm{M}^{-1}$. If $1/K_0^D$ is much greater than 1, then the affinity of M·D for actin is greater than the affinity of myosin alone for actin in step 1. This is possible but in the absence of other evidence seems unlikely.

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Registry No. ADP, 58-64-0.

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Enhancement of *Escherichia coli* RecA Protein Enzymatic Function by dATP[†]

Joseph P. Menetski[‡] and Stephen C. Kowalczykowski[*]
Department of Molecular Biology, Northwestern University Medical School, Chicago, Illinois 60611
Received November 29, 1988; Revised Manuscript Received March 22, 1989

ABSTRACT: The *Escherichia coli* recA protein has been shown to hydrolyze several nucleoside triphosphates in the presence of ssDNA. The substitution of dATP for rATP has significant effects on various recA protein biochemical properties. In the presence of dATP, recA protein can invade more secondary structure in native ssDNA than it can in the presence of rATP. The dATP–recA protein complex can compete more effectively with the *E. coli* ssDNA binding protein (SSB) for ssDNA binding sites compared with the rATP–recA protein complex. Finally, the rate of dATP hydrolysis stimulated by dsDNA is greater than the rate of rATP hydrolysis. These effects, in turn, are observed as alterations in the recA protein catalyzed DNA strand exchange reaction. In the absence of SSB protein, the rate of joint molecule and product formation in the DNA strand exchange reaction is greater in the presence of dATP than in the presence of rATP. The rate of product formation in the dATP-dependent reaction is also faster than the rATP-dependent reaction when SSB protein is added to the ssDNA before recA protein; the rate of rATP-dependent product formation is inhibited 10-fold under these conditions. This nucleotide, dATP, was previously shown to induce an apparent affinity of recA protein for ssDNA which is higher than any other NTP. These results suggest that the observed enhancement of enzymatic activity may be related to the steady-state properties of the high-affinity ssDNA binding state of recA protein. In addition, the data suggest that recA protein functions in NTP hydrolysis as a dimer of protein filaments and that the binding of ssDNA to only one of the recA filaments is sufficient to activate all recA protein molecules in the dimeric filament. The implications of this finding to the enzymatic function of recA protein are discussed.

RecA protein can catalyze the complete exchange of one single-stranded DNA (ssDNA)[1] molecule for a homologous strand within a duplex DNA molecule in a reaction called DNA strand exchange (Cox & Lehman, 1981a). This reaction requires the hydrolysis of a nucleoside triphosphate cofactor, typically rATP (Cox & Lehman, 1981a); however, the actual mechanistic requirement for rATP hydrolysis is unknown. Hypotheses for the role of rATP binding and hydrolysis in recA protein function include mediating the polar polymerization of recA protein on DNA (Griffith et al., 1984; Kowalczykowski et al., 1987), providing energy for concerted rotation of a recA protein filament to drive branch migration of heteroduplex joints (Schutte & Cox, 1987), and a way to

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[*] Author to whom correspondence should be addressed.

[‡] Present address: Laboratory of Molecular Biology, National Institutes of Health, Bethesda, MD.

[1] Abbreviations: PEP, phosphoenolpyruvate; ssDNA, single-stranded DNA; dsDNA, double-stranded DNA; etheno M13 DNA, modified M13 ssDNA containing 1,N^6-ethenoadenosine and 3,N^4-ethenocytidine residues; poly(dT), poly(thymidylic acid); poly(dA), poly(deoxyadenylic acid); poly(dA-dT), alternating copolymer of deoxyadenylic and thymidylic acid.

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