S. (1988) *Neuron 1*, 201-209.
Llinas, R., McGuinness, T., Leonard, C. S., Sugimori, M., & Greengard, P. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 3035-3039.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265-275.
McCaffery, C. L., & DeGennaro, L. J. (1986) *EMBO J. 5*, 3167-3173.
Morrow, J. S. (1989) *Curr. Opin. Cell. Biol. 1*, 23-29.
O'Farrell, P. H. (1975) *J. Biol. Chem. 250*, 4007-4021.
Petrucci, T. P., & Morrow, J. S. (1987) *J. Cell Biol. 105*, 1355-1363.
Petrucci, T. P., Mooseker, M. S., & Morrow, J. S. (1988) *J. Cell. Biochem. 36*, 25-35.
Rodriquez-Boulan, E., & Nelson, W. J. (1989) *Science 245*, 718-725.
Schiebler, W., Jahn, R., Doucet, J. P., Rothlein, J., & Greengard, P. (1986) *J. Biol. Chem. 261*, 8383-8390.
Shephard, E. G., De Beer, F. C., von Holt, C., & Hapgood, J. P. (1988) *Anal. Biochem. 168*, 306-313.
Speicher, D. W., Morrow, J. S., Knowles, W. J., & Marchesi, V. T. (1982) *J. Biol. Chem. 257*, 9093-9101.
Südhof, T. C., Czernik, A. J., Kao, H.-T., Takei, K., Johnston, P. A., Horiuchi, A., Kanazir, S. D., Wagner, M. A., Perin, M. S., DeCamilli, P., & Greengard, P. (1989) *Science 245*, 1474-1480.
Towbin, H., Stachelin, T., & Gordon, J. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 4350-4354.

Kinetics of the Interaction of 2′(3′)-*O*-(*N*-Methylanthraniloyl)-ATP with Myosin Subfragment 1 and Actomyosin Subfragment 1: Characterization of Two Acto·S1·ADP Complexes†

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ABSTRACT: We have used a fluorescent analogue of ATP, mantATP [2′(3′)-*O*-(*N*-methylanthraniloyl)-adenosine 5′-triphosphate; Hiratsuka, T. (1983) *Biochim. Biophys. Acta 742*, 496-508], and made a detailed kinetic study of the interaction of mantATP and mantADP with S1 and acto·S1. We have shown that these analogues behave like ATP and ADP, respectively. In addition, we have demonstrated that this analogue can distinguish between two acto·S1 complexes, the A-M·N (attached) and A·M·N (rigor-like) states [Geeves, M. A., Goody, R. S., & Gutfreund, H. (1984) *J. Muscle Res. Cell Motil. 5*, 351-361]. Previously, these two states were observed with a pyrene label on Cys 374 of actin. This isomerization can now be monitored at two spatially distinct sites on the ternary complex, indicative of a major conformational change in the ternary complex. Also, we have measured the rate of ADP dissociation from both A-M·N and A·M·N directly and shown these to differ by a factor of 1000. Thus the results presented here support the model of Geeves et al. and are consistent with the A-M·N to A·M·N transition being coupled to the force-generating event of the crossbridge cycle.

The mechanism of the ATPase reaction of myosin and its proteolytic subfragments (subfragment 1 and heavy meromyosin, S1[1] and HMM, respectively) has been studied by a wide variety of transient kinetic and isotope exchange techniques [for reviews, see Trentham et al. (1976), Taylor (1979), and Sleep and Smith (1981)]. A generally accepted mechanism of the first part of the ATPase reaction is shown in Scheme I.

Scheme I

$$M + T \underset{}{\overset{k_1}{\rightleftharpoons}} M\cdot T \overset{k_2}{\rightleftharpoons} M^*\cdot T \overset{k_3}{\rightleftharpoons} M^{**}\cdot D\cdot P$$

Step 1 is the formation of the binary collision complex (K_1 = 500 M^{-1}) followed by a rapid almost irreversible isomerization to the M*·ATP complex ($k_{+2} > 1000\ s^{-1}$, $K_2 > 10^7$) which is then reversibly hydrolyzed ($k_{+3} = 100\ s^{-1}$, $K_3 = 10$). (These kinetic and equilibrium constants are for pH 7.5, 20 °C, I = 0.15 M.) In subsequent steps (not shown) phosphate is released in the rate-limiting reaction, and then ADP is released in a relatively slow two-step reaction which is similar to a reversal of the two-step association of ATP.

Stopped-flow studies of the association of actin with S1 and S1-nucleotide complexes are compatible with a single-step binding reaction (Finlayson et al., 1969; White & Taylor, 1976; Marston, 1982; Konrad & Goody, 1982; Criddle et al., 1985). However, relaxation methods have shown this to be a three-step reaction: collision complex formation followed by two isomerization events (Scheme II).

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[1] Abbreviations: S1, myosin subfragment 1; mantATP, 2′(3′)-*O*-(*N*-methylanthraniloyl)adenosine 5′-triphosphate; mantdATP, 2′-deoxy-3′-*O*-(*N*-methylanthraniloyl)adenosine 5′-triphosphate; εATP, 1,N^6-etheno-ATP; DEDA-ATP, 2′(3′)-*O*-[*N*-[2-[[[5-(dimethylamino)-naphthyl]sulfonyl]amino]ethyl]carbamoyl]-ATP. The 5′-diphosphates are similarly abbreviated.

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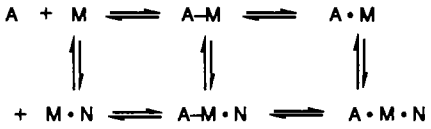
Scheme II

$$A + M \overset{K_o}{\rightleftharpoons} A\ M \overset{K_a}{\rightleftharpoons} A\text{–}M \overset{K_b}{\rightleftharpoons} A\cdot M$$

Scheme III



The first isomerization results in formation of an attached state ($K_o k_{+a} = 2 \times 10^6$ M^{-1} s^{-1}, $K_o K_a = 5 \times 10^4$ M^{-1}) followed by isomerization to the rigor-like complex ($K_b = 200$) (Coates et al., 1985; Geeves, 1989). The equilibrium constant of the second isomerization is particularly sensitive to the nucleotide bound to S1. In the absence of nucleotide it is 200, 10 in the presence of ADP, and $<10^{-2}$ in the presence of ATP. This isomerization is believed to be coupled to the force-generating event of muscle contraction (Geeves et al., 1984).

A central question in the mechanism of force generation by actomyosin is how the molecular events involved in nucleotide recognition and binding to S1 are coupled to the interaction between actin and S1. A mechanism based on the model of Geeves et al. (1984) is shown in Scheme III. This is essentially a combination of Schemes I and II with the collision complexes omitted.

The equilibrium constants have been recently assigned for the reactions in the presence of ADP (Geeves, 1989). Similar schemes have been proposed for other nucleotides but not all of the equilibrium constants have been measured (Geeves et al., 1984).

A principal feature of this scheme is that the isomerization of the acto·S1 complex from the attached state (A–M or A–M·N) to the "rigor-like" state (A·M or A·M·N) results in a change in the affinity of nucleotide for the complex. The A–M state binds nucleotide with an affinity similar to that of actin-free S1 whereas the affinity of nucleotide for A·M is many times weaker, the exact difference depending upon the nucleotide. Thus, the conformational change must influence both the actin binding site and the nucleotide binding site on S1 even though these sites are believed to be more than 3 nm apart (Botts et al., 1984).

Optical signals have been used to characterize each of the steps in Scheme III. Light scattering has been used to monitor the association between S1 or S1–nucleotide complexes and actin (Finlayson et al., 1969; White & Taylor, 1976). The fluorescence of a pyrene group covalently attached to Cys 374 of actin reports the A–M·N to A·M·N transition specifically (Coates et al., 1985; Geeves et al., 1986; Geeves & Jeffries, 1988), and intrinsic protein fluorescence reports nucleotide binding to S1 (Trentham et al., 1976). Fluorescent and chromophoric analogues of nucleotides have additionally been used to report events in the nucleotide binding steps. Most optically useful analogues of nucleotides have been modified on the adenine ring, which usually results in significant changes in the recognition of the analogue by S1 (Trentham et al., 1976).

Clearly, the use of a nucleotide analogue which more closely resembles ATP and which has optical signals which can report nucleotide binding would be useful. If in addition the analogue can distinguish the two types of nucleotide binding, then this would be useful in a study of the interrelation of the nucleotide and actin binding sites on S1. We report the use of such an analogue here.

Hiratsuka (1983a) has synthesized a range of nucleotides derivatized at the ribose moiety with the anthraniloyl or *N*-methylanthraniloyl groups and has shown that their steady-state catalysis parameters are very similar to those of the physiological nucleotides for a range of enzymes, including HMM with mantATP. The corresponding guanine nucleotide analogues also closely mimic the parent nucleotide with guanine nucleotide binding proteins (Eccleston et al., 1989; Neal et al., 1990). Hiratsuka subsequently used the adenine derivatives to investigate the nucleotide binding site of myosin by a combination of fluorescence and photoaffinity labeling techniques (Hiratsuka, 1983b, 1984a,b), and Cremo et al. (1990) have described the fluorescence perturbations occurring on binding to S1 in detail. The use of mantATP to measure processes occurring in skinned muscle fibers has been recently reported, and the analogue was shown to support contraction and relaxation to a similar extent as ATP (Ferenczi et al., 1989).

Cremo et al. (1990) have shown that mantADP exists as an equilibrium mixture of the 2′-isomer (35%) and the 3′-isomer (65%). We have therefore synthesized the *N*-methylanthraniloyl derivative of 2′-deoxy-ATP (mantdATP) in which isomerization cannot occur and compared its interaction with subfragment 1 with that of the ATP derivative.

We report here a detailed study of the interaction of mantATP with S1 and acto·S1 using transient kinetic methods. We demonstrate that in most respects the analogue behaves like ATP and the fluorescence of the analogue increases 2.6-fold on binding to S1. We also show that the analogue can distinguish between the A–M·N and A·M·N states. The pyrene label on actin can also distinguish these two complexes, and therefore, the isomerization can be monitored independently at two spatially distinct sites on the ternary complex. In addition, the rates of ADP dissociation from both A–M·N and A·M·N are measured and shown to differ by a factor of 1000. The implication of these results for the acto·S1 mechanism is discussed.

Materials and Methods

The preparation of myosin S1 and F-actin was as described by Weeds and Taylor (1975) and Lehrer and Kerwar (1972), respectively.

N-Methylanthraniloyl derivatives of ATP, ADP, dATP, and dADP were prepared by reaction with *N*-methylisatoic anhydride (Molecular Probes) as described by Hiratsuka (1983a) except that after reaction the analogues were purified on a DEAE-cellulose column. The column was eluted with a linear gradient of 10 mM to 0.8 M triethylammonium bicarbonate. The fluorescent nucleotides eluted after, and well resolved from, the unreacted nucleotide. The pooled fractions were evaporated to dryness in vacuo, and remaining triethylamine was removed by three additions and evaporations of methanol. Possible deacylation during this procedure was excluded on the basis of the lack of any parent nucleotide (<1%) in the solutions as determined by HPLC (see below). The 5′-diphosphate analogues were also prepared by hydrolysis of the 5′-triphosphates with myosin S1.

The *N*-methylanthraniloyl derivatives of ATP and ADP had absorption spectra, fluorescence spectra, and thin-layer chromatography properties identical with those described by Hiratsuka (1983a). All of the analogues were further characterized by hydrolysis in 50 mM NaOH for 15 min at room temperature when they were hydrolyzed to the parent nucleotide and *N*-methylanthranilic acid.

Stopped-flow measurements were carried out on a Hi-Tech Scientific SF3L instrument which has been characterized by Geeves et al. (1986). For fluorescence measurements, excitation light at 290 and 366 nm from a 75-W Hg arc lamp was obtained with a monochromator and a UG11 filter, and em-

itted light was monitored through a KV 393 cutoff filter. For light scattering measurements, light at 407 nm direct from the monochromator was monitored through the KV 393 filter. For each experiment the data shown are the average of four to six consecutive pushes of the stopped-flow. The data were fitted with a nonlinear least-squares procedure to either a single or double exponential (Edsall & Gutfreund, 1983), and the fitted curves are superimposed on the experimental data. All concentrations stated refer to concentrations after mixing.

Quenched-flow experiments were performed on the instrument described by Eccleston et al. (1985) except that reactants were loaded into both syringes rather than the final sample loop. A 200-μL total reaction mixture was ejected into 200 μL of 10% perchloric acid at 4 °C and rapidly adjusted to pH 4 by the addition of 100 μL of 4 M sodium acetate. After centrifugation, the supernatents were analyzed by HPLC on a Whatman Partisil 10 SAX column (250 × 4.6 mm) eluting at 2 mL min^{-1} with 0.6 M $NH_4H_2PO_4$, pH 4.0, containing 15% methanol. The eluant was monitored with a Gilson Model 121 fluorometer and the signal recorded with a Hewlett Packard 3390A integrator.

Fluorescence excitation and emission spectra were recorded on an SLM 8000S spectrofluorometer. Spectroscopic titrations of S1 with mantADP were made on the same instrument. To a stirred solution of 5 μM S1 (2 mL) was continuously added a solution of 300 μM mantADP at 12.5 μL min^{-1}. Fluorescence was monitored with excitation at 362 nm and emission at 440 nm. After data from a titration in the absence of S1 were subtracted, the data were analyzed as described by Geeves (1989).

Steady-state ATPase rates were measured with a pH stat at 20 °C, pH 8.0. The solution contained 2 mM Tris, 5 mM $MgCl_2$, 125 mM KCl, and 2 mM ATP or mantATP. Rates were measured with 10 μM S1 and varying concentrations of F-actin, titration being with 1 mM KOH. All other measurements were made in a buffer of 50 mM sodium cacodylate adjusted to pH 7.0 with HCl, 0.1 M KCl, and 5 mM $MgCl_2$ to allow a direct comparison with rate constants obtained with ATP (Millar & Geeves, 1988).

Results

Fluorescence Change on Binding mantADP to S1. The fluorescence emission spectra of 5 μM mantADP in the presence and absence of 30 μM S1 showed a 2.6-fold enhancement of fluorescence on formation of the complex, similar to the result described by Cremo et al. (1990). On the basis of the value of the K_D of S1 for mantADP (see below), greater than 99% of the nucleotide was bound to protein in the presence of S1. The emission maximum was at 440 nm in both cases.

The fluorescence excitation spectra showed maxima at 362 nm for unbound mantADP and at 368 nm for S1·mantADP. An additional excitation maximum at 294 nm occurred with S1-mantADP, resulting from energy transfer from tryptophan to mantADP since the excitation spectrum of mantADP closely overlaps the emission spectrum of tryptophan in S1. This is particularly useful for some of the experiments described below when measurements of complexes in the presence of excess fluorescent nucleotide are being made. Since the excitation of free nucleotide is almost zero at 290 nm, the bound nucleotide can be excited preferentially and so increase the signal to background ratio relative to measurements at 366 nm.

Similar results were obtained with mantdADP. The enhancement was 2.1-fold, the excitation maxima for free and bound nucleotide were 360 and 368 nm, respectively, and the emission maxima were at 442 nm in both cases.

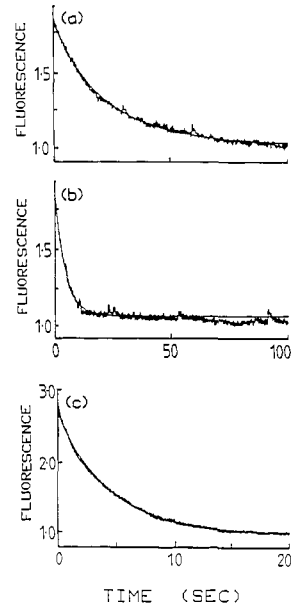


FIGURE 1: Stopped-flow record of the steady-state rate of mantATP hydrolysis by S1 and the dissociation rate of mantADP at 20 °C. All solutions contained 50 mM sodium cacodylate adjusted to pH 7.0 with HCl, 0.1 M KCl, and 5 mM $MgCl_2$. The solid lines are best fits to single exponentials. (a) One syringe contained 3 μM S1 and 15 μM mantATP, and the other contained 1 mM ATP. The reaction was initiated within 1 min of preparing the S1–mantATP solution. k_{obs} = 0.042 s^{-1}. (b) As (a) except that the reaction was initiated 5 min after preparing the S1–mantATP solution. k_{obs} = 0.22 s^{-1}. (c) One syringe contained 5 μM S1 and 5 μM mantADP, and the other contained 1 mM ATP. k_{obs} = 0.23 s^{-1}.

Displacement of mant Nucleotides from Complexes with S1. The steady-state intermediate of the S1–mantATPase was formed by mixing S1 with a 5-fold excess of mantATP. Within 1 min, this solution was rapidly mixed with a large excess of ATP in a stopped-flow apparatus and fluorescence emission observed (Figure 1a). A decrease in fluorescence intensity occurred which could be fitted to a single exponential with a first-order rate constant of 0.042 s^{-1}. This rate represents the displacement of mant nucleotide from the steady-state complex, and since the dissociation of mantATP from S1 is $<10^{-3}$ s^{-1} (see below), this rate represents the turnover rate of the mantATPase and is similar to that of the ATPase (0.05 s^{-1}). When the solution of S1 and mantATP was allowed to stand for 5 min before being mixed with excess ATP, a faster process occurred with a rate constant of 0.22 s^{-1} (Figure 1b). This was shown to be the dissociation of mantADP from a S1·mantADP complex since a process of identical rate constant was observed on mixing a solution containing stoichiometric concentrations of S1 and mantADP with excess ATP (Figure 1c). With the improved signal to noise ratio of the latter experiment, a small (<10%) component of a faster process was also observed. This reaction was examined over a range of ratios of S1:mantADP from 1:1 to 1:20. Under all conditions >90% of the observed reaction could be fitted to a single exponential with an observed rate constant of 0.22 s^{-1}, and the fast phase remained too small to be analyzed.

Binding of mant Nucleotides to S1. The rate of binding of mantADP to S1 was measured under pseudo-first-order conditions (Figure 2a,b). At low concentrations (<20 μM) of mantADP, measurements were made with excitation at 366 nm, but at higher concentrations (>20 μM) excitation was at 290 nm (see above). At 20 μM mantADP, the reaction was measured with excitation at both wavelengths, and identical fluorescence time courses were observed.

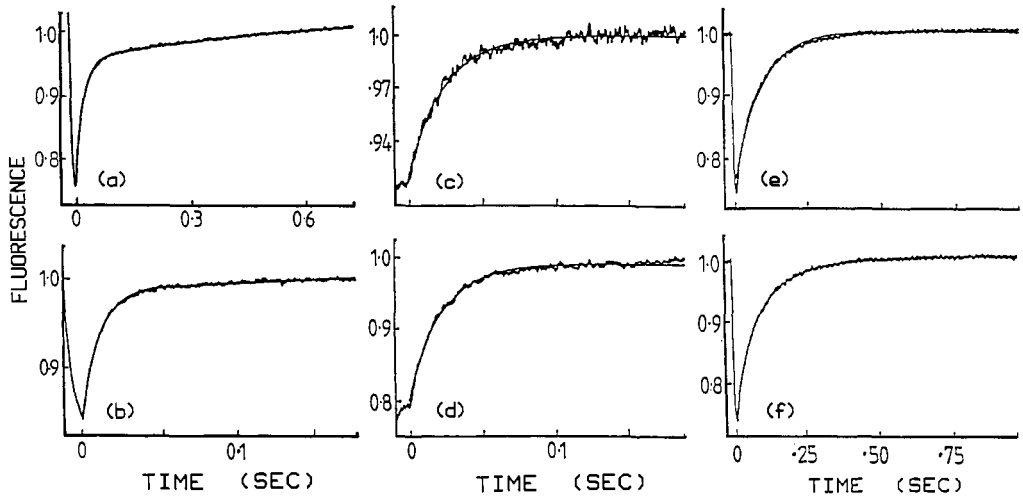


FIGURE 2: Stopped-flow records of the binding of mantADP and mantATP to S1. (a) Binding of mantADP to S1 at 20 °C. One syringe contained 2 μM S1 and the other 20 μM mantADP. Excitation was at 290 nm. k^1_{obs} = 41.5 s^{-1}; k^2_{obs} = 3.5 s^{-1}; A_1/A_2 = 3.8. (b) As (a) except that mantADP was 40 μM. k_{obs} = 69.9 s^{-1}. (c) Binding of mantATP to S1 at 20 °C. One syringe contained 2 μM S1 and the other 20 μM mantATP. Excitation was at 366 nm. k_{obs} = 43.1 s^{-1}. (d) As (c) except that excitation was at 290 nm. k_{obs} = 41.3 s^{-1}. (e) Binding of mantATP to S1 at 4 °C. One syringe contained 2 μM S1 and the other 60 μM mantATP. Excitation was at 290 nm. The solid line is the best fit to a single exponential. k_{obs} = 10.3 s^{-1}. (f) As (e) except the solid line is the best fit to a double exponential. k^1_{obs} = 20.1 s^{-1}; k^2_{obs} = 6.3 s^{-1}; A_1/A_2 = 1.2.

At concentrations of mantADP up to 20 μM, there was an indication of a biphasic reaction (observable at both excitation wavelengths). The slow phase was linearly dependent upon [mantADP], with a second-order rate constant of 0.62 × 10^6 M^{-1} s^{-1}. The amplitude of the slow phase was 58% at 2.5 μM and decreased until it could not be detected above 20 μM. The observed rate of the fast phase, and the single phase at higher [mantADP], was linearly dependent on [mantADP] and gave a second-order rate constant of 2.3 × 10^6 M^{-1} s^{-1} (Figure 3a). Combination of this second-order association rate constant with the dissociation rate constant of mantADP from S1 gives a value for the equilibrium dissociation constant of 0.1 μM. Measurement of the dissociation constant obtained by titrating mantADP into a solution of S1 and monitoring fluorescence gave a value of 0.2 μM (data not shown). These results show that the rate of dissociation of mantADP from S1 is approximately 10-fold slower than that of ADP, resulting in mantADP binding to S1 10-fold tighter than ADP.

The rate of binding of mantATP to S1 was measured under the same solution and optical conditions as described for mantADP. At 20 μM mantATP, again no difference could be detected in the fluorescence changes observed on excitation at 290 or 366 nm.

At 20 °C, the fluorescence change could be fitted to a single exponential over the range of mantATP concentrations used (2.5–100 μM). A plot of observed rate constant against [mantATP] shows an approximately linear dependence up to 40 μM (second-order rate constant of 2.0 × 10^6 M^{-1} s^{-1}) and then levels off and may even decrease. The observed rate constant at 100 μM mantATP was 80 s^{-1}. Deterioration in the signal to noise precluded measurements at higher concentrations.

The result is similar to that observed for ATP binding to S1 when the reaction is followed with intrinsic protein fluorescence under identical conditions (Millar & Geeves, 1988). For ATP a second-order rate constant of 1.0 × 10^6 M^{-1} s^{-1} was obtained with a maximum observed rate of 90 s^{-1}. The result with ATP has been attributed to the presence of two steps in the reaction; rapid ATP binding and a slower ATP hydrolysis step (90 s^{-1}) both contributing a protein fluorescence change (Johnson & Taylor, 1978). The two phases cannot be distinguished at 20 °C, but their presence is suggested by

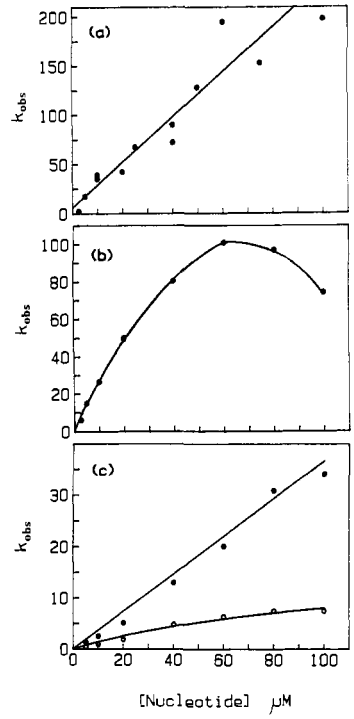


FIGURE 3: Concentration dependence of the observed processes on mixing mantADP and mantATP with S1. Data from experiments as shown in Figure 2 were analyzed as the best fit to single or double exponentials. (a) Dependence of the observed rate constant of the fluorescence change on the concentration of mantADP at 20 °C. The data above 20 μM mantADP were from reaction traces fitted to a single exponential, and the data below 20 μM mantADP are the fast phases of the reaction traces fitted to double exponentials. The solid line is the best fit to a straight line. k = 2.3 × 10^6 M^{-1} s^{-1}. (b) Dependence of the observed rate constant of the fluorescence change fitted to a single exponential on the concentration of mantATP at 20 °C. The solid line is a smooth curve drawn through the data points. (c) Dependence of the observed rate constants obtained from a fit to a double exponential on the concentration of mantATP at 4 °C. (●) Rate constants of the fast phase; (○) rate constants of the slow phase. The upper solid line is the best fit of the fast phase rate constants to a straight line. k ≈ 3.7 × 10^5 M^{-1} s^{-1}.

the unusual dependence of both the observed rate and amplitude on [ATP]. Accurate analysis of the fluorescent am-

Table I: Summary of Rate Constants of the Interaction of mant Nucleotides and ATP with Subfragment 1:[a]

$$M + ATP \overset{K_1}{\rightleftharpoons} M{\cdot}ATP \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} M{\cdot}ATP^* \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} M{\cdot}ADP{\cdot}P_i \overset{k_4}{\rightleftharpoons}$$

	20 °C				1 °C	4 °C
	ATP	mantATP	mantdATP	mantADP	ATP	mantATP
K_1k_2 (M^{-1} s^{-1})	1.0×10^6 [b]	3.2×10^6	1.5×10^6	2.3×10^6		3.7×10^5
k_{-2} (s^{-1})				0.22		
$k_3 + k_{-3}$ (s^{-1})	90[b]	~80	~100	>200	5.5[b]	<20
k_{cat} (s^{-1})	0.05	0.04	0.07			
k_p (s^{-1})					7[c]	14

[a] K_1k_2 = observed second-order rate constant of nucleotide binding to S1 at concentrations of nucleotide where the observed rate constant was proportional to nucleotide concentration. k_{-2} = observed first-order rate constant from displacement experiments. $k_3 + k_{-3}$ = limiting rate constant of fluorescence signal at high concentrations of nucleotide. k_{cat} = turnover rate by displacement from the steady-state intermediate. k_p = rate constant of phosphate burst from quenched-flow experiments. Data are from this work except where indicated. [b] Millar & Geeves, 1988. [c] Sleep & Taylor, 1976.

plitudes of the reaction with mantATP is difficult because the free mantATP contributes to the background signal even when exciting the protein fluorescence. However, by analogy with ATP we propose that the observed process of 80 s^{-1} at 100 μM mantATP represents the cleavage step.

At lower temperatures evidence for two phases in the mantATP binding reaction is stronger. Figure 2e,f shows mantATP binding to S1 at 4 °C. The reaction can now be seen to be biphasic. The fast phase was linearly dependent upon [mantATP] over the range measured (second-order rate constant 0.37×10^6 M^{-1} s^{-1}), and the slow phase deviated from a straight line fit (Figure 3c). We were unable to measure the observed rate over a wide enough concentration range to define a complete concentration dependence, but we estimate the maximum rate to be <20 s^{-1}. This can be compared to a value of 5.5 s^{-1} measured for the maximum rate of the protein fluorescence change on ATP binding to S1 at 1 °C (Millar & Geeves, 1988).

The rate of the cleavage step was measured directly in a quenched-flow experiment. A solution of 14 μM mantATP was mixed with a solution of 100 μM S1 at 4 °C and quenched at times between 7 ms and 100 ms in the single push mode and between 100 and 5 s in the pulsed flow mode. A rapid formation of mantADP occurred followed by a slower phase. The rapid formation of mantADP occurred with a rate constant of 14 s^{-1} and an end point of 66% (data not shown) compared to a rate of 7 s^{-1} in the case of ATP at 3 °C, 40 mM KCl, pH 6.9 (Sleep & Taylor, 1976). This supports the argument that the slow phase observed in the fluorescence measurements is the cleavage step. Further support comes from the binding of mantADP to S1 which shows only a single phase with linear concentration dependence between 20 and 100 μM and a rate constant similar to that of the fast phase of mantATP binding to S1 (Figure 3a). A fluorescence change similar to the slow phase is also seen on mantATP binding to acto·S1 (see below).

The amplitude of 66% of the rapid phase of the hydrolysis of mantATP by excess S1 gives a value for K_3 (the equilibrium constant between M*·T and M**·D·P; Scheme I) of 1.9 compared to a value of 1.0 for ATP under the conditions of Sleep and Taylor (1976). Since the observed rate of mantADP formation is equal to $k_3 + k_{-3}$ and the ratio of [mantATP]/[mantADP] at the end of the burst reaction is equal to k_3/k_{-3}, values of k_3 and k_{-3} of 9.2 s^{-1} and 4.8 s^{-1}, respectively, can be calculated. When a solution of 14 μM mantATP and 100 μM S1 was made 2 mM in ATP after 5 s at 4 °C and then quenched with perchloric acid at 5 min, less than 1% mantATP could be detected in the reaction mixture. Under these conditions, the ratio [mantATP]:[mantADP] is given by $k_{-2}k_{-3}/k_3k_4$ (Bagshaw & Trentham, 1973) so that an upper limit of the rate of dissociation of mantATP from S1 (k_{-2}) of 10^{-3} s^{-1} is obtained.

Interaction of mant Deoxynucleotides with S1. As shown by Cremo et al. (1990) mant nucleotides exist as an equilibrium mixture of the 2′- and 3′-isomers. In order to exclude the possibility that such a process gives rise to any of the fluorescent signals associated with the interaction of mant ribonucleotides with S1 described above, most of the experiments were repeated with mantdATP and mantdADP. No signifieant differences were observed in amplitudes and rate constants (Table I). However, it should be noted that although the release of mantADP and mantdADP occurred at similar rates (0.2 and 0.5 s^{-1}, respectively), both processes showed evidence of biphasicity, <10% of a fast phase with mantADP and <10% of a slow phase with mantdADP, although in both cases the processes became more monophasic at higher concentrations of nucleotide. The result with mantdADP excludes the biphasic nature of the process with mantADP being due to the 2′- and 3′-mant derivatives being displaced at different rates.

Actin Activation of the mantATPase. The ability of actin to activate the hydrolysis of mantATP by S1 was measured by steady-state rate measurements with a pH stat. In a solution containing 10 μM S1 and 2 mM ATP or 2 mM mantATP, the rate of hydrolysis in both cases was proportional to the concentration of actin over the range 0–30 μM. However, the activation of mantATP hydrolysis by actin was approximately 50% of that of the activation of ATP hydrolysis.

Binding of mantATP to Acto·S1. The rate of the mantATP-induced dissociation of acto·S1 can be monitored by following changes in light scattering, and such a reaction at 20 °C is shown in Figure 4a. The observed rate was linearly related to the concentration of mantATP up to 50 μM with a second-order rate constant of 3×10^6 M^{-1} s^{-1} (Figure 4d) which is similar to the value observed for ATP. At concentrations above 50 μM there was evidence of a deviation from the linear dependence observed at lower concentrations. A hyperbolic dependence of the dissociation rate has been reported for ATPγS under these conditions (Goody & Hofmann, 1980) and for ATP at temperatures below 1 °C (Millar & Geeves, 1983). We were unable to use high enough concentrations of mantATP to reliably establish the characteristics of a hyperbolic dependence although the fitted line in Figure 4d gives $K_{0.5}$ = 70 μM and k_{max} = 388 s^{-1}.

The same reaction was monitored by observing mant fluorescence with excitation at either 290 nm (Figure 4b) or 366 nm (Figure 4c). The concentration dependence of the reaction (Figure 4d) was similar to that of mantATP binding

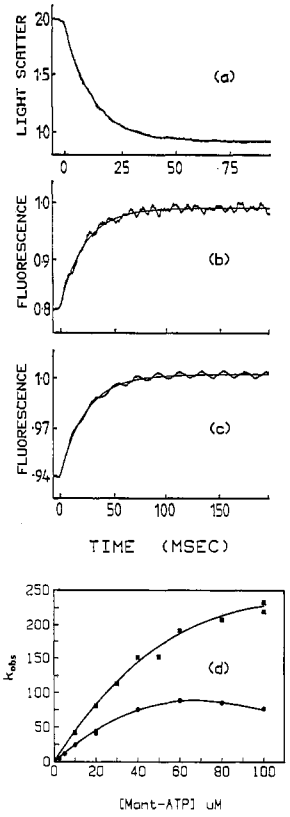
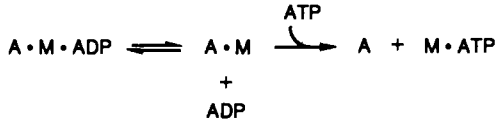


FIGURE 4: Binding of mantATP to acto·S1 at 20 °C. (a) Stopped-flow record of the binding of mantATP to acto·S1. One syringe contained 2 μM acto·S1 and the other 20 μM mantATP. Light scattering was observed. k_{obs} = 81.1 s^{-1}. (b) As (a) except that fluorescence emission was observed with excitation at 290 nm. k_{obs} = 43.7 s^{-1}. (c) As (a) except that fluorescence emission was observed with excitation at 366 nm. k_{obs} = 41.0 s^{-1}. (d) The dependence of the observed rate constants from experiments as above on the concentration of mantATP. (■) Light scattering; (●) fluorescence. The solid lines are smooth curves drawn through the data points.

to S1 with a limiting value of 80 s^{-1}, supporting the previous conclusion that this signal arises from the hydrolysis step. This is the same conclusion as from studies monitoring protein fluorescence upon the interaction of acto·S1 with ATP (Millar & Geeves, 1988).

Binding of mantADP to acto·S1. The affinity of ADP for acto·S1 can be assessed by measuring the ADP inhibition of the ATP-induced dissociation of acto·S1 (White, 1977; Siemankowski & White, 1984):



At low concentrations of ATP such that the dissociation of ADP from acto·S1 remains at equilibrium throughout the time course of the experiment, the dependence of the observed rate of acto·S1 dissociation is defined by

$$k_{obs} = k_o/(1 + [ADP]K_A)$$

where k_o is the observed rate constant in the absence of ADP and K_A is the equilibrium association constant of ADP for acto·S1.

Such an experiment with mantADP is shown in Figure 5 where it can be seen that the rate of dissociation decreases as the concentration of mantADP increases. The best fit of the experimental data to the above equation gave a value for K_A of 8.3 × 10^3 M^{-1}, which is similar to the value for the binding of ADP to acto·S1 (Geeves, 1989). A value of 4.3 × 10^3 M^{-1} was obtained for mantdADP.

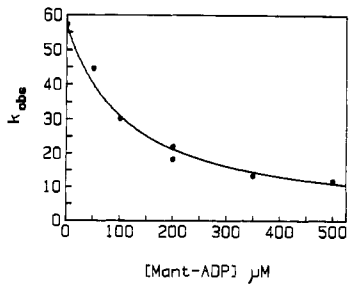


FIGURE 5: Affinity of mantADP for acto·S1. Stopped-flow records of light scattering were obtained on mixing 5 μM acto·S1 with 50 μM ATP in the presence of increasing concentrations of mantADP (added to the acto·S1 syringe). In all cases the observed process could be fitted to a single exponential. A plot of the observed rate constant as a function of mantADP concentration is shown. The solid line is the best fit to the equation $k_{obs} = k_o/(1 + [mantADP]K_A)$, which gave k_o = 58 s^{-1} and K_A = 8.3 × 10^3 M^{-1}.

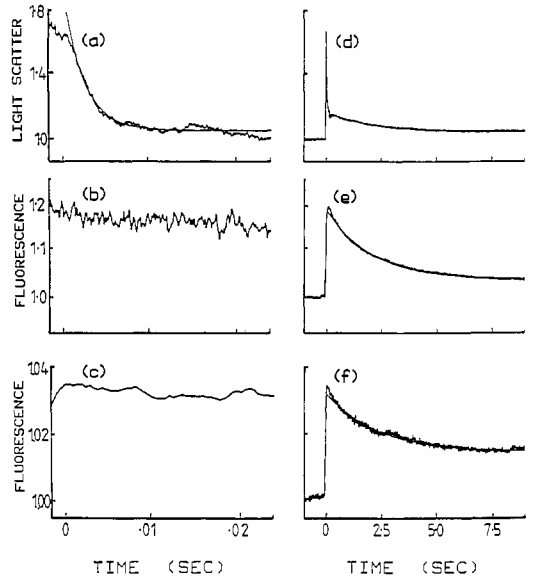
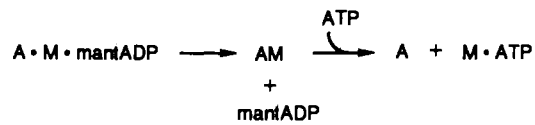


FIGURE 6: Stopped-flow records of the displacement of mantADP from the acto·S1·mantADP ternary complex. (a) One syringe contained 5 μM acto·S1 and 100 μM mantADP and the other syringe contained 1 mM ATP. Light scattering was observed. k_{obs} = 393 s^{-1}. (b) As (a) except that fluorescence was observed with excitation at 290 nm. (c) As (a) except that fluorescence was observed with excitation at 366 nm. (d–f) The above experiments repeated with the reaction monitored over a longer time scale. The values of k_{obs} are 0.48 s^{-1}, 0.53 s^{-1}, and 0.44 s^{-1}, respectively. The solid lines are the best fits to single exponentials.

The rate of mantADP dissociation from the acto·S1·mantADP complex can be examined by measuring the limiting rate of the ATP-induced dissociation of the complex at high concentrations of ATP:



The limiting rate of the ATP-induced dissociation of acto·S1 has been estimated as 5000 s^{-1} (Millar & Geeves, 1983).

Figure 6a shows that, on mixing a solution of the acto·S1·mantADP complex with 1 mM ATP, the observed rate of dissociation of acto·S1 was 393 s^{-1}. This rate constant was independent of increasing ATP concentration, which defines this process as the dissociation of mantADP from acto·S1·mantADP. The equivalent value for ADP is >500 s^{-1}. Observing the same reaction by monitoring mant fluorescence with either excitation at 290 nm (Figure 6b) or 366 nm (Figure 6c) shows that no change in fluorescence occurs corresponding

to the displacement of mantADP from the ternary complex. The light scattering signal demonstrates that mantADP is binding to acto·S1 and competes with ATP for the same binding site. Therefore, the mantADP must be binding to acto·S1 with no perturbation of its fluorescence.

Examining the mantADP dissociation reaction on a slower time scale by monitoring light scattering or either of the two fluorescence signals revealed the presence of a slower component of the dissociation/displacement reaction (Figure 6d–f). In each case the observed rate constant was 0.5 s^{-1}. This suggests the presence of a second population of acto·S1·mantADP complex from which mantADP dissociates at 0.5 s^{-1}. In the case of light scattering, the signal had 15% of the amplitude of the fast component. The ternary complex was formed by mixing actin, S1, and mantADP, and the concentrations of these three components were such that the presence of acto·S1 and S1·mantADP binary complexes could not be eliminated. However if acto·S1 were present, it would be dissociated by ATP at the concentration used at a rate of greater than 1000 s^{-1} and then would contribute only to the light scattering signal and not the fluorescence signal. If S1·mantADP was present, the mantADP would be displaced at a rate of 0.22 s^{-1} (see Figure 1) and would only contribute to the fluorescence signal. What is observed here is a slow process which can be observed both by light scattering and by fluorescence. The observed rate of the fluorescence change is twice the rate observed in the absence of actin, and although this signal may contain a contribution from the dissociation of any S1·mantADP present, the increase in the observed rate and the presence of the light scattering change at the same rate show that the observed process is the dissociation of mantADP from an acto·S1·mantADP ternary complex.

The experiments shown in Figure 6 were repeated with mantdADP. Again, light scattering showed a fast phase followed by a slower process, and fluorescence only monitored the slow phase.

Discussion

On binding mantATP to S1 there is evidence that the change of fluorescence monitors two distinct steps. At 4 °C, this is manifested in a biphasic process. Although the data at 20 °C, can be fitted to a single exponential, the lack of linearity of the observed rate constant with nucleotide concentration also suggests a two-step process as described under Results. Analysis of the 4 °C data shows a fast process which is linearly dependent upon nucleotide concentration up to 100 μM; this also occurs with mantADP binding to S1 at 20 °C, and we therefore attribute it to the nucleotide binding event. The second-order rate constant for this process and the temperature dependence of the rate are not those expected for a diffusion-controlled reaction, and the process therefore probably includes the formation of a collision complex which is not directly observable. We attribute the second fluorescence change to the hydrolysis step since it occurs at a similar rate as the independently measured hydrolysis of the nucleotide by S1. Also, the limiting rate constant of the fluorescence change on binding mantATP to S1 and acto·S1 at 20 °C is similar.

The fluorescence changes accompanying nucleotide binding and hydrolysis by S1 and acto·S1 have been studied previously with both the intrinsic protein fluorescence (Johnson & Taylor, 1978; Garland & Cheung, 1979; Millar & Geeves, 1988) and the fluorescence of nucleotide analogues (Tesi et al., 1988; Smith & White, 1985; Rosenfeld & Taylor, 1984). The advantage of the analogue used here is that both the fluorescence and the change in fluorescence of mant nucleotides are much larger than those of the intrinsic protein fluorescence, and second, the spectral overlap between the mant group absorbance and the tryptophan emission allows the nucleotide binding reaction to be followed by fluorescence energy transfer. This means that the signal to noise ratio remains tolerable to 100 μM nucleotide concentrations without having to add a collisional quencher to reduce the fluorescence of the free nucleotide relative to the bound nucleotide (Rosenfeld & Taylor, 1984).

Two phase changes in fluorescence upon nucleotide binding to S1 have been reported by the authors mentioned above using protein fluorescence and fluorescent nucleotide analogues. The experiments presented here were performed under the same conditions as the experiments of Millar and Geeves (1988), and the results are compatible with those obtained for ATP binding by these authors. Table I compares the two sets of data in relation to model I. It can be seen that mantATP closely mimics the behavior of ATP unlike either the 1,N^6-ethenoadenosine or 1,N^6-(ethenoaza)adenosine nucleotides (Rosenfeld & Taylor, 1984; Smith & White, 1985; Tesi et al., 1988).

A more complex mechanism has been proposed by Rosenfeld and Taylor (1984) to account for their data with ϵATP. In their experiments (at a much lower ionic strength than that used here) a two-phase fluorescence change was observed for both ϵATP and ϵADP binding to S1, and the rates of both fluorescence changes for ϵATP binding were much faster than that of the hydrolysis event. To account for their data, two S1–nucleotide complexes are required on a branched pathway. An alternative explanation was proposed by Tesi et al. (1988), who examined this same reaction at low ionic strength in the presence of ethylene glycol and also observed two fluorescence transients when [ϵATP] > S1 but only a single phase when S1 > [ϵATP]. These authors proposed a model involving two types of nucleotide binding sites on S1. We repeated both the mantATP and mantADP binding experiments with [S1] ≫ [nucleotide] and still observed biphasic fluorescence changes. The majority of the data presented here can however be adequately explained by the simpler model of Millar and Geeves (1988), which was based on the earlier work of Johnson and Taylor (1978).

The one observation which does not fit this model simply is the binding of mantADP to S1, which shows two fluorescence transients at low concentrations when either mantADP or S1 is in excess. As the slower signal disappears at [mantADP] > 20 μM, it cannot readily be explained by heterogeneity of the protein. Similarly, heterogeneity of the nucleotide (such as the 2′- and 3′-isomers) would not explain the loss of one phase at high concentrations as the two isomers would remain in a fixed ratio at all times. The branched pathway of Rosenfeld and Taylor (1984) could account for this result with one pathway dominating at high ADP concentrations.

When excess mantATP was mixed with acto·S1, only a single phase of fluorescence change was observed, and this was slower than the rate of acto·S1 dissociation observed by light scattering. This suggests that there is no fluorescence change corresponding to the binding of mantATP to acto·S1 although over most of the nucleotide concentration range used there was only a factor of 2 difference between the two signals. The presence of two phases in the fluorescence signal which are not resolved cannot therefore be excluded.

The studies using mant nucleotides made from deoxyribose precursors showed little difference in their behavior compared to the ribose nucleotides. In addition to demonstrating the similarity of the ribose and deoxyribose derivatives, the ex-

periments suggest that the presence of mixed 2′- and 3′-derived ribose isomers is not responsible for any of the phenomena observed and therefore any difference in the behavior of the two isomers is very small.

The results presented here show that in most respects mantATP is a good analogue of ATP. The rate of mantATP binding to S1, its rate of hydrolysis, and the steady state turnover rate are indistinguishable from those of ATP. Similarly, the rate of the mantATP-induced dissociation of acto·S1, the affinity of mantADP for acto·S1, and the actin activation of the S1 ATPase rate are all very similar to those of ATP. These results are supported by the observation that mantATP supports tension development and relaxation in single skinned rabbit psoas fibers to a similar extent as does ATP (Ferenczi et al., 1989). The only significant difference observed in the behavior of mant-labeled nucleotides is that the affinity of mantADP for S1 is 10 times tighter and the dissociation rate is 10 times slower than the equivalent rates for ADP. Tighter binding of mant nucleotides occurs with several proteins and may represent an interaction of the mant group with a hydrophobic group in the nucleotide binding site (Hiratsuka, 1983a; J. F. Eccleston and M. A. Geeves, unpublished results). Tighter binding of mantATP may occur but would not be apparent in the studies reported here as the ATP dissociation rate cannot be measured directly. The tighter binding of mantADP to S1 is not seen in the case of acto·S1 where the observed binding constants for ADP and mantADP are very similar. This difference in the nature of the binding site on S1 and acto·S1 will be discussed more fully in the following paragraphs.

Having established that in most respects mantATP is a good analogue of ATP, the information available from the fluorescence of the mant group can be considered. If the reaction for mantADP binding is monophasic and the reaction for mantATP binding and hydrolysis is biphasic, then it is of interest to know the relative fluorescence of myosin-bound mantATP, mantADP·P_i, and mantADP. Analysis of the relative amplitudes of the two phases of the reaction with mantATP is not useful as the two phases cannot be cleanly separated under any of the conditions used in this study. A single turnover experiment ([S1] > [mantATP]) will demonstrate the difference in fluorescence between the steady-state complex (a mixture of M·mantATP, M·mantADP·P_i, and M·mantADP) and the final fluorescence which will be entirely M·mantADP assuming insignificant dissociation of mantADP (K_D = 0.1 μM). Such an experiment showed that the fluorescence decreased from the steady state to the final signal by ~10%. Similarly, a comparison of the observed amplitude for 40 μM mantATP and mantADP binding to 3 μM S1 at 20 °C where a single phase is seen for both nucleotides showed the ATP reaction to have a 10% larger amplitude. Also, Cremo et al. (1990) have observed that the analogues of the M·mantADP·P_i states (formed by chemical cross-linking or by trapping with vanadate) have an approximately 10% increase in fluorescence over that of the M·mantADP complex. Thus it appears that the M·mantADP·P_i complex has a higher fluorescence than either M·mantATP or M·mantADP.

In addition to demonstrating the suitability of the mant nucleotides as analogues of ATP and ADP and confirming earlier studies on the elementary steps associated with nucleotide binding and hydrolysis by S1, the work reported here provides new information on the interaction between nucleotides and acto·S1. The experiment of Figure 5 demonstrates that mantADP competes with ATP binding to acto·S1 and the affinity of mantADP is similar to that of ADP. However, the displacement of mantADP from the ternary complex with acto·S1 demonstrates the presence of two populations of bound mantADP. The first, the majority complex, is bound with an affinity of 8.3×10^3 M^{-1} and dissociates at 393 s^{-1}. Significantly, this bound mantADP dissociates without any change in fluorescence. The second population dissociates at 0.5 s^{-1} and does so with a decrease in fluorescence. The relative amplitudes of the light scattering changes in Figure 6 define the relative sizes of the two populations. The fitted amplitudes are in the ratio of 5.6:1, but the fast phase has to be corrected for the amplitude lost in the dead time of the apparatus (0.9 ms; Millar, 1984), which gives a ratio closer to 8.0:1. This observation is significant in relation to the model of the interaction of actin with myosin and myosin–nucleotide complexes proposed by Geeves et al. (1984) (Scheme III). This model proposed the presence of two acto·S1·ADP complexes, and these were later observed by Geeves and Jeffries (1988) and Geeves (1989) using actin which had a fluorescent pyrene group covalently attached to Cys 374. Under conditions similar to those used here the two complexes were shown to be present in a ratio of 10:1. No signal was obtained when ADP dissociated from the minority species, but ADP dissociated from the majority complex at >500 s^{-1}. Although the displacement of ADP from the minority A–M·D complex could not be observed directly, the model required the displacement rate to be similar to the rate of ADP displacement from S1·ADP (~2 s^{-1}). The direct observation of mantADP displacement from the minority complex observed here confirms this prediction and allows the rate consant to be measured directly.

This direct observation of mant·ADP displacement from the two acto·S1 complexes is significant for two reasons; it confirms the existence of two acto·S1·ADP complexes and demonstrates that the transition from A–M·D to A·M·D changes the nature of the nucleotide–protein interaction. mantADP is bound relatively tightly to A–M with a displacement rate only twice that of mantADP release from S1 alone. The environment of the mant fluorophore in A–M·D is also similar to that in M·D; therefore, actin binding itself does not change the interaction between ADP and S1. Only the formation of the rigor-like A·M·D changes the environment of the mant group and accelerates the rate of ADP dissociation. The mant group is therefore sensitive to the transition between the A–M·D and A·M·D states. The fluorescence of the pyrene group covalently attached to actin is sensitive to the same transition. Therefore, this isomerization of the ternary complex is perturbing the environment of both Cys 374 on actin and the nucleotide binding site on S1 even though these two sites have been shown to be 3 nm apart by fluorescence energy transfer measurements (Botts et al., 1984). The isomerization must therefore represent a major change in the conformation of the complex.

The lack of a change in fluorescence between mantADP in solution and in the A·M·D. complex is surprising in view of the large enhancement in fluorescence when bound to S1. Enhancements also occur when mantATP binds to tyrosine kinase or oxoglutarate dehydrogenase (M. A. Geeves and J. F. Eccleston, unpublished results) and when mant-guanine nucleotides bind to elongation factor Tu (Eccleston et al., 1989) and to p21N-ras (Neal et al., 1990). However, mantdGDP binds tightly to elongation factor Tu without showing any enhancement of fluorescence. A series of steady-state and time-resolved fluorescence measurements and circular dichroism studies showed that in this complex the mant fluorophore shows no interaction with the protein but is freely exposed to the solvent (Eccleston et al., 1990). Presumably,

a similar situation occurs in the A·M·D. complex.

We have also made preliminary experiments with new analogues of ATP and ADP, DEDA-ATP and DEDA-ADP, in which there is a longer spacer arm between the ribose and fluorophore (Cremo et al., 1990). These analogues also behave very similar to the parent nucleotides in their interaction with S1 and acto·S1. However, displacement of DEDA-ADP from the ternary complexes with S1 and actin shows a biphasic process when light scattering is being observed but only a slow process when fluorescence is being observed.

The model of the actomyosin ATPase proposed by Geeves et al. (1984) requires that the binding of actin to form the A–M·N state does not significantly change the nature of the nucleotide binding site (i.e., its formation does not change the affinity of nucleotide for S1 significantly) and that the A–M·N to A·M·N isomerization is a major change in the conformation of the complex. The isomerization observed by the pyrene signal could represent a small local perturbation of the environment around Cys 374 alone. The results presented here and the observation that the isomerization involves a substantial molar volume change in the presence and absence of ADP (Coates et al., 1985; Geeves, 1989) exclude this argument. The work presented therefore supports the model proposed by Geeves et al. (1984) and is consistent with the A–M·N to A·M·N transition being coupled to the force-generating event of the crossbridge cycle.

Registry No. ATP, 56-65-5; ATPase, 9000-83-3; 3′-mantATP, 85287-56-5; 2′-mantATP, 130799-34-7; 3′-mantADP, 85287-55-4; 2′-mantADP, 125902-32-1; mantdATP, 128113-53-1.

References

Bagshaw, C. R., & Trentham, D. R. (1973) *Biochem. J. 133*, 323–328.

Bagshaw, C. R., Eccleston, J. F., Eckstein, F., Goody, R. S., Gutfreund, H., & Trentham, D. R. (1974) *Biochem. J. 141*, 351–364.

Botts, J., Takashi, R., Turgeson, P., Hozumi, T. Muhlrad, A., Mornet, D., & Morales, M. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 2060–2064.

Coates, J. H., Criddle, A. H., & Geeves, M. A. (1985) *Biochem. J. 232*, 351–356.

Cremo, C. R., Neuron, J. M., & Yount, R. G. (1990) *Biochemistry 29*, 3309–3319.

Criddle, A. H., Geeves, M. A., & Jeffries, T. E. (1985) *Biochem. J. 232*, 343–349.

Eccleston, J. F., Dix, D. B., & Thompson, R. C. (1985) *J. Biol. Chem. 260*, 16237–16241.

Eccleston, J. F., Kanagasabai, T. F., Molloy, D. P., Neal, S. E., & Webb, M. R. (1989) in *The Guanine Nucleotide Binding Proteins: Common Structural and Functional Properties* (Bosch, L., Kraal, B., & Parmeggiani, A., Eds.) NATO Advanced Science Institutes Series A, Vol. 165, pp 87–97, Plenum, New York.

Eccleston, J. F., Woodward, S. K. A., Kanagasabai, T. F., & Martin, S. R. (1990) *Biophys. J. 57*, 50a.

Edsall, J. T., & Gutfreund, H. (1983) *Biothermodynamics*, Wiley, New York.

Ferenczi, M. A., Woodward, S. K. A., & Eccleston, J. F. (1989) *Biophys. J. 55*, 441a.

Finlayson, B., Lymn, R. W., & Taylor, E. W. (1969) *Biochemistry 8*, 811–819.

Garland, F., & Cheung, M. C. (1979) *Biochemistry 18*, 5281–5289.

Geeves, M. A. (1989) *Biochemistry 28*, 5864–5871.

Geeves, M. A., & Jeffries, T. E. (1988) *Biochem. J. 256*, 41–46.

Geeves, M. A., Goody, R. S., & Gutfreund, H. (1984) *J. Muscle Res. Cell Motil. 5*, 351–361.

Geeves, M. A., Jeffries, T. E., & Millar, N. C. (1986) *Biochemistry 25*, 8454–8458.

Goody, R. S., & Hofmann, W. (1980) *J. Muscle Res. Cell Motil. 1*, 101–115.

Hiratsuka, T. (1983a) *Biochim. Biophys. Acta 742*, 496–508.

Hiratsuka, T. (1983b) *J. Biochem. 93*, 875–882.

Hiratsuka, T. (1984a) *J. Biochem. 96*, 155–162.

Hiratsuka, T. (1984b) *J. Biochem. 96*, 147–154.

Johnson, K. A., & Taylor, E. W. (1978) *Biochemistry 17*, 3432–3442.

Konrad, M., & Goody, R. S. (1982) *Eur. J. Biochem. 128*, 547–555.

Lehrer, S. S., & Kerwar, G. (1972) *Biochemistry 11*, 1211–1217.

Marston, S. B. (1982) *Biochem. J. 203*, 453–460.

Millar, N. C. (1984) Ph.D. Thesis, University of Bristol, U.K.

Millar, N. C., & Geeves, M. A. (1983) *FEBS Lett. 160*, 141–148.

Millar, N. C., & Geeves, M. A. (1988) *Biochem. J. 249*, 735–743.

Neal, S. E., Eccleston, J. F., & Webb, M. R. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 3562–3565.

Rosenfeld, S. S., & Taylor, E. W. (1984) *J. Biol. Chem. 259*, 11920–11929.

Siemankowski, R. F., & White, H. D. (1984) *J. Biol. Chem. 259*, 5045–5053.

Sleep, J. A., & Taylor, E. W. (1976) *Biochemistry 15*, 5813–5817.

Sleep, J. A., & Smith, S. J. (1981) *Curr. Top. Bioeng. 11*, 239–286.

Smith, S. J., & White, E. W. (1985) *J. Biol. Chem. 260*, 15156–15162.

Taylor, E. W. (1979) *CRC Crit. Rev. Biochem. 6*, 102–164.

Tesi, C., Travers, F., & Barman, T. (1988) *Biochemistry 27*, 4903–4908.

Trentham, D. R., Eccleston, J. F., & Bagshaw, C. R. (1976) *Q. Rev. Biophys. 9*, 217–281.

Weeds, A. G., & Taylor, R. S. (1975) *Nature (London) 257*, 54–56.

White, H. D. (1977) *Biophys. J. 17*, 40a.

White, H. D., & Taylor, E. W. (1976) *Biochemistry 15*, 5818–5826.