The effect of nucleotide upon a specific isomerization of actomyosin subfragment 1

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The binding of actin to myosin subfragment 1 (S1) has been shown to occur as a two-step reaction [Coates, Criddle & Geeves (1985) Biochem. J. **232**, 351–356]. In the first step actin is weakly bound and the second step involves the complex isomerizing to a more tightly bound state. This isomerization can be followed specifically by monitoring the fluorescence of actin that has been covalently labelled with N-(pyren-1-yl)-iodoacetamide at Cys-374 [Geeves, Jeffries & Millar (1986) Biochemistry **25**, 8454–8458]. We report here that the presence of nucleotides and nucleotide analogues affects the equilibrium between the strongly bound and weakly bound states (referred to as K_2). In the presence of ATP, [γ-thio]ATP or ADP and vanadate a value of approx. $< 10^{-2}$ was estimated for K_2. In the presence of PP_i or ADP a value of approx. 2.3 or 10 respectively was obtained. An increase in KCl concentration or the presence of 40 % ethylene glycol was found to decrease K_2 in the presence of ADP. The data presented here are consistent with the two-step binding model proposed by Geeves, Goody & Gutfreund [(1984) J. Muscle Res. Cell Motil. **5**, 351–361], where it was suggested that the transition between weakly bound and strongly bound states is closely associated with the force-generating event in whole muscle.

INTRODUCTION

Force generation in muscle is the result of a dynamic interaction between actin and myosin that is modulated by the presence of nucleotide. The molecular event that results in force generation is believed to be related to a change in the nature of the interaction between actin and myosin. The effect of nucleotide and nucleotide analogues on this interaction is therefore of interest.

Myosin–nucleotide complexes have been classified as weak actin-binding states (for example myosin–ATP and myosin–ADP–phosphate) or strong actin-binding states (for example myosin–ADP and myosin) (Eisenberg & Greene, 1980). A general model was proposed by Geeves et al. (1984) that could explain these different actin-binding properties:

$$A + MN \underset{}{\overset{K_1}{\rightleftharpoons}} A\text{–}MN \underset{}{\overset{K_2}{\rightleftharpoons}} AMN \qquad (1)$$
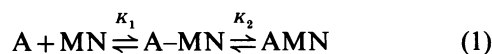
where A represents actin, M represents myosin, N represents nucleotide and K_n is the equilibrium constant for the nth step of a reaction.

In this model actin binds in a two-step reaction. In the first step actin binds to form a weakly bound ternary complex, with an association constant of 10^3–10^4 M^{-1}. This may then isomerize to a ternary complex where actin is strongly bound. It was proposed that the equilibrium constant for the isomerization is dependent upon the nucleotide present in the S1 nucleotide-binding site. The value of the equilibrium constant, K_2, of this step dictates whether the ternary complex is in either the strong or the weak actin-binding state. For example if $K_2 > 1$ then the strong complex predominates; if $K_2 < 1$

then the weak complex predominates. Related models have been proposed by Trybus & Taylor (1982) and Shriver & Sykes (1981).

Interactions between actin and S1 in solution can be monitored by using changes in the light-scattering signal that is observed when actin and S1 associate. An alternative and more sensitive method is to use actin that has been labelled at Cys-374 with N-(pyren-1-yl)iodo-acetamide (actin*; Molecular Probes, Eugene, OR, U.S.A.). The fluorescence of the pyrene label is quenched by 70–80 % when S1 binds to actin* (Kouyama & Mihashi, 1981). Criddle et al. (1985) demonstrated that the presence of the label on actin had little effect upon the equilibrium and dynamics of actin–S1 interactions. Coates et al. (1985) investigated the dynamics of actin*–S1 interactions in the absence of nucleotide by using both light-scattering and fluorescence. They demonstrated that the light-scattering and fluorescence signals reported different events and that this was consistent with the two-step binding model (as in eqn. 1) where light-scattering monitored the association reaction and fluorescence monitored the subsequent isomerization. The use of pyrene-labelled actin therefore allows independent observation of the isomerization and dissociation steps.

If it were possible to achieve sufficiently high acto-S1 concentration such that addition of nucleotide would induce essentially no dissociation, then any change in the fluorescence signal would report the transition of a fraction of the acto-S1 from the strongly attached low-fluorescence state to the weakly attached high-fluorescence state. Experimentally it can be difficult to

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achieve high enough protein concentrations to ensure that no dissociation can take place.

Geeves et al. (1986) demonstrated that at low ionic strength the addition of 250 μM-ATP to 50 μM-acto-S1 resulted in only 30% dissociation as judged by light-scattering measurements. However, the fluorescence was identical with that observed for free actin, indicating that the bound acto-S1 (70%) was all in the high-fluorescence A–MN state. Thus the equilibrium constant [AMN]/[A–MN] was estimated to be < 10⁻².

In the present paper we report a series of experiments that use the above approach to determine the equilibrium constant of the isomerization step in the presence of a range of nucleotides and nucleotide analogues.

MATERIALS AND METHODS

Proteins

Myosin subfragment 1 (S1) was prepared by a chymotryptic digest of rabbit skeletal-muscle myosin, as described by Weeds & Taylor (1975). The two isoenzymes S1.A1 and S1.A2 were separated by ion-exchange chromatography on a DEAE-cellulose column, elution being with a linear gradient of 0–0.1 M-KCl/imidazole buffer, pH 7.0. The purity of the separated isoenzymes was confirmed by SDS/polyacrylamide-gel electrophoresis. The S1.A1 isoenzyme was used throughout. F-actin was prepared according to the method described by Lehrer & Kerwar (1972). Protein concentrations were calculated by using M_r 115000 and $A_{280}^{1\,cm}$ 7.9 cm⁻¹ for S1 (Margossian & Lowey, 1978) and M_r 42000 and $A_{280}^{1\,cm}$ 11.08 cm⁻¹ for actin (West et al., 1967). Pyrene-labelled actin (actin*) was prepared as described by Criddle et al. (1985).

Materials

ADP (monopotassium salt) and ATP[S] were obtained from Boehringer Mannheim, Lewes, East Sussex, U.K., and the ADP was used without further purification. ATP[S] was purified by ion-exchange chromatography on a DEAE-cellulose column, elution being with a linear gradient of 0–0.6 M-triethylammonium bicarbonate buffer, pH 7.6. The purity of the ATP[S] was assayed by h.p.l.c. The concentrations of the nucleotides were determined by using the molar absorption coefficient at 259 nm of 15.4 M⁻¹·cm⁻¹. A 0.1 M stock solution of Na₄P₂O₇ (AnalaR; BDH Chemicals, Poole, Dorset, U.K.) was prepared in an appropriate experimental buffer and kept at 4 °C before use.

A 0.1 M stock solution of trisodium vanadate was also prepared in an appropriate experimental buffer. The pH of the solution was adjusted to 10 with either 1 M-NaOH or 1 M-HCl, after which it was heated at 100 °C for approx. 5 min. The vanadate solution was kept at 4 °C before use.

Fluorescence measurements

Fluorescence measurements were performed on a Perkin–Elmer LS-5B luminescence spectrophotometer. The temperature of the cell holder was maintained at 20 ± 0.2 °C by an LKB 2219 Multitemp 11 thermostatic circulator.

For pyrene fluorescence measurements excitation was at 365 nm and emission at 407 nm, with 2.5 nm excitation and emission slit widths. Light-scattering measurements were made above 400 nm in order to be free of

interference from the pyrene signal. The excitation monochromator was routinely set at 413 nm and the emission monochromator at 420 nm, also with 2.5 nm slits.

The quench of the pyrene fluorescence on actin by S1 binding was routinely 70–80%. This is greater than the 68% that we previously reported with a Farrand fluorimeter.

Data analysis

Data obtained from steady-state titrations were corrected for dilution effects upon adding S1 and then fitted by using a non-linear least-squares fitting routine to the following quadratic equation:

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

where $[A]_0$ is the starting concentration of actin, $[M]_0$ is the total concentration of S1 added, K_d is the dissociation constant and α is the fractional saturation of actin by 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 experimentally derived values of $[A]_0$, F_0, F and $[M]_0$ and yielded values for K_d and F_∞.

RESULTS

ADP

The experimental approach that was undertaken to investigate the equilibrium between the strong and the weak binding states in the presence of ADP was the most straightforward to perform.

The dissociation constant of MgADP for acto-S1 is approx. 200 μM (100 mM-KCl/5 mM-MgCl₂/10 mM-Tris, pH 8.0, at 20 °C), and this is reasonably insensitive to temperature, pH, and ionic strength (White, 1977). The dissociation constant of S1–MgADP for actin was determined by Geeves & Gutfreund (1982) to be 1.27 μM (100 mM-KCl/5 mM-MgCl₂/50 mM-imidazole, pH 7.0, at 20.7 °C). Thus addition of 1 mM-ADP to 50 μM-S1 and 5 μM-actin* would result in saturation of actin with both S1 and ADP. Such an experiment is illustrated in Fig. 1 and shows only a small transient change in light-scattering signal on addition of ADP, this being due to the presence of contaminating ATP. The fluorescence signal shows the same small transient change but it does not return to the signal observed for acto-S1 alone. If pyrene fluorescence is monitoring the isomerization step then the change in fluorescence would represent a shift in occupancy from the strongly to the weakly attached state on binding ADP. The equilibrium constant can be estimated from the fluorescence signal. If actin fluorescence is F_A, acto-S1 fluorescence is F_{AS} and the fluorescence in the presence of ADP is F_D, then the fraction in the low-fluorescence state is $(F_A - F_D)/(F_A - F_{AS}) = 0.91$. Light-scattering shows no dissociation, and therefore $K_2 = [AMD]/[A–MD] = 0.91/0.09 = 10.1$.

Since Coates et al. (1985) reported that in the absence of nucleotide K_2 was decreased by either the presence of ethylene glycol or increased ionic strength, the experiment was repeated in the presence of 40% (v/v) ethylene glycol or increased salt concentration (see Table 1). Ethylene glycol decreased K_2 by 4-fold, and altering the
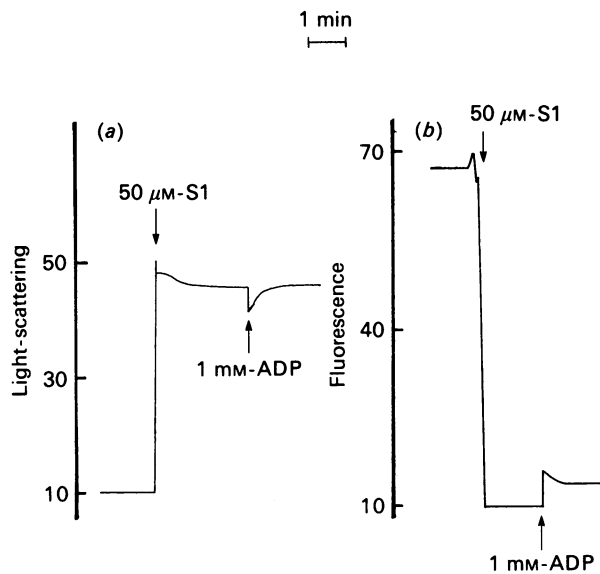
Fig. 1. Fluorescence and light-scattering changes on ADP binding to acto-S1

(a) 90° light-scattering on adding 50 μM-S1 to 5 μM-actin* followed by 1 mM-ADP. (b) As (a), but with fluorescence being monitored. The experiments were performed in 0.1 M-KCl/2 mM-MgCl$_2$/50 mM-cacodylate buffer, pH 7.0.

Fig. 2. Fluorescence changes on adding ATP[S] to acto-S1

Trace A, fluorescence changes on adding 7 μM-S1 to 5 μM-actin* followed by 250 μM-ATP[S]. Trace B, fluorescence changes on adding 70 μM-S1 to 5 μM-actin* followed by 1 mM-ATP[S]. The experiments were performed in 2 mM-MgCl$_2$/10 mM-Mops buffer, pH 7.0, containing 1 mM-dithiothreitol.

ionic strength by increasing KCl from 0.1 M to 0.3 M decreased K_2 by a similar amount.

ATP[S]

ATP[S] is an analogue of ATP that is hydrolysed slowly by S1 (0.25 s^{-1}; Bagshaw et al., 1972). Goody & Hofmann (1980) reported that ATP[S] caused complete dissociation of acto-S1 at low protein concentration (50 mM-KCl / 5 mM-MgCl$_2$ / 100 mM-Tris, pH 8.0, at 23 °C). The affinity of actin for S1·ATP[S] is expected to be of the same order as for S1·ATP. In a buffer containing 2 mM-MgCl$_2$ and 10 mM-imidazole, pH 7.0, the affinity of actin for S1·ATP is of the order of 15 μM (Chalovich & Eisenberg, 1982). Therefore addition of ATP[S] to an acto-S1 solution at protein concentrations greater than 15 μM is expected to lead to incomplete dissociation. Fig. 2 shows that addition of 250 μM-ATP[S] to 7 μM-S1 and 5 μM-actin caused the fluorescence signal to return to that observed for free actin, until hydrolysis of ATP[S] had been completed. At this protein concentration over 80% dissociation of the acto-S1 complex is expected. At 70 μM-S1 and 5 μM-actin the
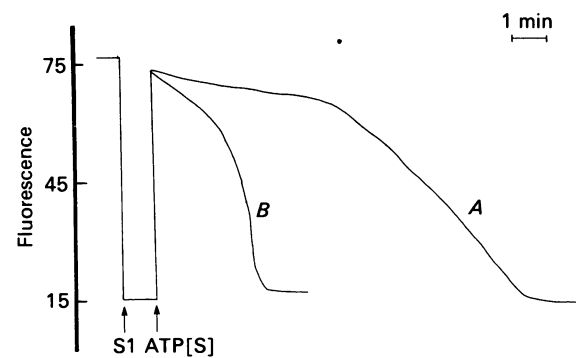
Table 1. Effect of KCl and ethylene glycol on K_2 in the presence of ADP

The experiment was performed in 2 mM-MgCl$_2$/50 mM-cacodylate buffer, pH 7.0, containing 1 mM-ADP.

[KCl] (M)	K_2
0.0	> 50
0.1	10.0
0.3	2.5
0.1 + 40% ethylene glycol	2.7

addition of 1 mM-ATP[S] caused the same transient fluorescence signal change as in the low-[S1] case. The addition of ATP[S] to either acto-S1 sample resulted in a rapid decrease in light-scattering followed by an increase on the same time scale as the change in fluorescence. The magnitude of the light-scattering decrease was decreased at the higher S1 concentration, but the extent of the subsequent recovery of light-scattering was not reproducible (90–110% of the decrease in duplicate measurements). It is possible that aggregation of the proteins occurs more readily in the presence of ATP[S] than in the presence of ATP, where reproducible light-scattering signals were obtained. The variability of these results does not allow a reliable estimation of the extent of dissociation caused by ATP[S]. However, in an equivalent experiment with ATP the change in light-scattering signal indicated that at 50 μM-S1 and 5 μM-actin 70% of the actin remained bound to the S1·ATP complex (Geeves et al., 1986). Thus, assuming that S1·ATP[S] binds actin at least as tightly as does the S1·ATP complex, then the low-fluorescence strongly attached state is not significantly occupied under these conditions. Given the uncertainty about the extent of dissociation, we estimate the equilibrium constant between the two attached states [AM·ATP[S]]/[A–M·ATP[S]] to be less than 0.05.

ADP·vanadate

Since there is similarity in the structure of vanadate and phosphate, the M·ADP·V$_i$ complex (where V$_i$ represents inorganic vanadate) can be used as a stable analogue of the steady-state M·ADP·P$_i$ intermediate ($t_{\frac{1}{2}}$ 1–2 days, cf. 10 s for M·ADP·P$_i$; Goodno & Taylor, 1982). Goodno & Taylor (1982) demonstrated that the M·ADP·V$_i$ complex had the same properties as the myosin–products intermediate, M·ADP·P$_i$, and that actin increased the rate of dissociation of vanadate from this complex.

Since vanadate will not readily bind to acto-S1, the following approach was taken; an S1·ADP·V$_i$ complex was formed by incubation of the S1 with 1 mM-ADP and 1 mM-vanadate for at least 30 min. The complex was
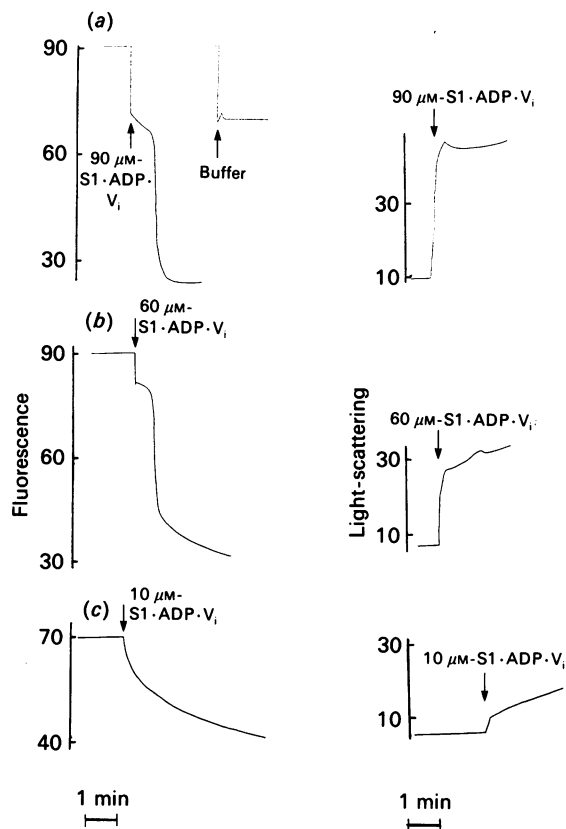
Fig. 3. Fluorescence and light-scattering changes on adding S1·ADP·V$_i$ to actin

(a) Fluorescence and 90° light-scattering changes on adding 90 μM-S1·ADP·V$_i$ (final concn.) to 5 μM-actin*. The fluorescence changes on adding an equivalent volume of buffer are shown. (b) As (a), but with 60 μM-S1·ADP·V$_i$ (final concn.). (c) As (a), but with 10 μM-S1·ADP·V$_i$ (final concn.). The experiments were performed in 2 mM-MgCl$_2$/10 mM-Hepes buffer, pH 7.5.

Fig. 4. Effect of PP$_i$ on the ability of S1 to quench actin* fluorescence

(a) Fluorescence titration of S1 into 5 μM-actin* in the presence of 0.5 mM-PP$_i$ (●), 2.0 mM-PP$_i$ (■) and 4.0 mM-PP$_i$ (○). (b) The maximum percentage quench of the titration of actin with S1 as a function of PP$_i$ concentration. The experiments were performed in 2 mM-MgCl$_2$/10 mM-Hepes buffer, pH 7.5.

added to 5 μM-actin* so that the final concentration of S1 present was as shown in Fig. 3. Light-scattering and fluorescence signal changes were monitored. The experiment was performed at three S1 concentrations. At 10 μM-S1 a small but immediate increase in light-scattering signal suggested that actin and S1 had formed a complex quickly. This was followed by a much slower increase in scattering. As the S1 concentration was increased the amplitude of the fast phase increased and that of the slow phase decreased. At 90 μM-S1 essentially only the fast phase was observed. The actin fluorescence decreased slowly on the addition of 10 μM-S1·ADP·V$_i$. At higher S1 concentrations the fluorescence showed three phases: a rapid decrease, which could be accounted for by the dilution effect, followed by a lag phase and then a further decrease. We attribute these changes to actin binding rapidly to S1·ADP·V$_i$ with no significant change in fluorescence followed by a gradual loss of vanadate resulting in the slow signal changes in both light-scattering and fluorescence. Recent studies have shown that in the presence of vanadate near-u.v. light can photochemically modify S1 (Grammer & Yount, 1987; Cremo et al., 1988). This modification takes 3–4 min to complete and could therefore contribute to the
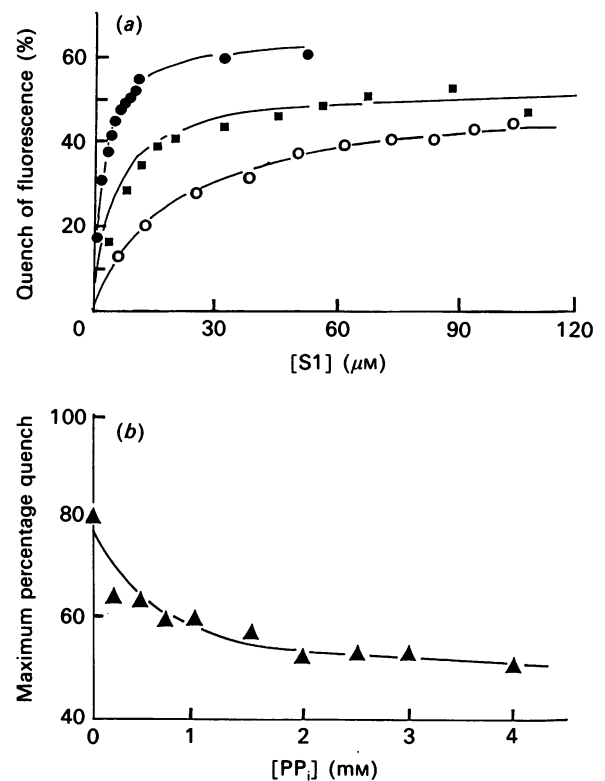
signals observed during the slow phase. The estimation of the equilibrium constant (K_2) relies upon the signal change observed immediately after adding S1·ADP·V$_i$ to the cuvette and therefore any photomodification of S1 will not affect this estimation. Thus it can be deduced that the change in fluorescence that reports the isomerization occurred after association of the S1·ADP·V$_i$ with actin. Therefore the equilibrium constant [AS1·ADP·V$_i$]/[A–S1·ADP·V$_i$] is of the same order as that with ATP and ATP[S], i.e. < 10^{-2}.

PP$_i$

The dissociation constant of actin from S1·PP$_i$ and acto-S1 from PP$_i$ at 4 °C and ionic strength 0.17 is 500 μM (Greene & Eisenberg, 1980).

It is difficult to saturate acto-S1 with PP$_i$ and actin with S1·PP$_i$, and therefore a more elaborate experimental approach was required compared with the previous experiments. Several actin–S1 titrations were performed in the presence of increasing concentration of PP$_i$. The change in fluorescence signal as S1 was bound to actin* in the presence of 0.25–4.0 mM-PP$_i$ was monitored. A set of titration curves was obtained and an apparent dissociation constant obtained as described in the Materials and methods section. Fig. 4(a) shows three titration curves at different PP$_i$ concentrations. These

curves allow an estimate of the maximum extent to which the fluorescence was quenched and the affinity of S1 for actin at each PP_i concentration. It was found that, as the concentration of PP_i increased, then the maximum fluorescence quench was smaller and the affinity of S1 for actin was decreased. A plot of percentage maximum fluorescence quench against PP_i concentration is shown in Fig. 4(b), and this allowed an estimate of the fluorescence of acto-S1·PP_i at infinite PP_i concentration. The data suggest a fluorescence quench of 55% at infinite PP_i concentration compared with 79% at zero PP_i. This result is consistent with an equilibrium for $[AM·PP_i]/[A-M·PP_i]$ calculated to be $55/24 = 2.3$. This may be a maximum value for K_2 in the presence of PP_i if the affinity of acto-S1 for PP_i is weaker than estimated here.

DISCUSSION

The interpretation of the results presented here is dependent upon the assignment of the fluorescence change to the isomerization step of the two-step binding reaction and this step alone. It is appropriate therefore to examine the validity of the argument that assigns the fluorescence to this step. The work of Coates et al. (1985) established that the pyrene label monitored an event that was different from the association reaction monitored by light-scattering. Geeves et al. (1986) demonstrated that addition of ATP to acto-S1 could result in complete reversal of the fluorescence change observed on binding actin to S1 without causing dissociation, thereby establishing that no significant fluorescence change occurs on the dissociation step itself. The question that remains to be addressed is whether any significant fluorescence change takes place on binding nucleotide to the ternary complex independently of the isomerization step. As the nucleotide-binding site of S1 has been shown to be 1–2 nm away from the position of Cys-374 on actin (Botts et al., 1984), the pyrene group will not be directly affected by the proximity of the nucleotide. Therefore any change in pyrene fluorescence must result from a nucleotide-induced change in the protein stucture around Cys-374. Recent kinetic studies by M. A. Geeves (unpublished work) have shown that ADP binds rapidly and reversibly to acto-S1, but the ADP-induced fluorescence change (shown in Fig. 1) occurs at a rate of < 10 s^{-1}, confirming that binding of nucleotide does not produce a fluorescence change. Of course the possibility of there being more than a single protein conformation change cannot be eliminated. Having established that pyrene fluorescence is monitoring an isomerization of acto-S1, we must now consider if this isomerization is the same structural change in the absence of nucleotide, in the presence of ATP and for the range of nucleotides and nucleotide analogues used here. The work of Coates et al. (1985) demonstrated that the equilibrium constant K_2 was greatly decreased by increasing KCl concentrations and by the presence of 40% ethylene glycol. The data in Table 1 demonstrate that the equilibrium constant estimated in the presence of ADP is affected in a similar way; thus increasing KCl from 0.1 to 0.3 M decreased K_2 by 2–3-fold in the absence of nucleotide and by 4-fold in the presence of ADP. The presence of 40% ethylene glycol decreased K_2 by 10-fold in the absence of nucleotide and by 4-fold in the presence of ADP. This suggests that the structural change monitored by pyrene fluorescence

has the same properties in the presence and in the absence of ADP. The binding of actin to the other myosin–nucleotide complexes used here is so weak that the experiment could only be performed at very low ionic strength and in the absence of ethylene glycol, and therefore the effect of these parameters could not be determined.

Estimates for the value of K_2 with the various nucleotides/analogues is shown in Table 2. All of the results presented are consistent with those myosin–nucleotide complexes that bind weakly to actin only occupying the A–M·N high-fluorescence state. This state can be attained directly by binding the myosin–nucleotide complex to actin or by binding the nucleotide to acto-S1, which then induces the A·M·N-to-A–M·N transition. Those complexes that bind more tightly to actin (M·ADP, M·PP$_i$) can occupy both states, and in the absence of nucleotide Coates et al. (1985) previously demonstrated that only the A·M·N state is significantly occupied. These data are consistent with the model of the actomyosin ATPase mechanism proposed by Geeves et al. (1984).

In this model values for the equilibrium constants of the isomerization were calculated from the overall binding constants. The values measured here differ by up to a factor of 10 from the previously calculated values, but the principle of the model is unaffected. Two-step binding is a property of all myosin–nucleotide complexes, and the affinity of a myosin–nucleotide complex is determined principally by the value of K_2. The binding of ATP to actomyosin results in only the A–M·N state being occupied, and as ATP is hydrolysed and the products are sequentially released then the actomyosin reverts gradually to the tightly bound A·M state. The data are consistent with the proposal that the transition between the two states could be closely linked with the force-generating event.

The ionic-strength-dependence of K_2 has implications for those actomyosin–nucleotide complexes that can only be studied at very low ionic strength. The extent to which such complexes can occupy the A·M·N state may be overestimated compared with the results that would be observed at normal physiological ionic conditions. The error may be small for M·ATP where K_2 is $< 10^{-2}$, but for M·PP$_i$ the correction could be quite large. The major unresolved question is the value of the equilibrium

Table 2. **Effect of nucleotide/nucleotide analogue on K_2 in the absence of KCl**

Experimental details can be found in the appropriate Figure legends.

Nucleotide/analogue	K_2
PP$_i$	2.3
V$_i$	$< 10^{-2}$
ATP[S]	< 0.05
ATP*	$< 10^{-2}$
ADP (0.1 M-KCl)	10
No nucleotide†	300

* Geeves et al. (1986). Conditions: 0.0 M-KCl, pH 7.0, at 20 °C.

† Coates et al. (1985). Conditions: 0.1 M-KCl, pH 7.0, at 20 °C.

constant K_2 for $M \cdot ADP \cdot P_i$. No direct information is available on this state, but if the model proposed by Geeves *et al.* (1984) is correct and the transition from the $A-M \cdot N$ state to the $A \cdot M \cdot N$ state is closely related to the force-generating event, then a value of K_2 closer to 1 than 10^{-2} might be expected for $M \cdot ADP \cdot P_i$.

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