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The use of actin labelled with *N*-(1-pyrenyl)iodoacetamide to study the interaction of actin with myosin subfragments and troponin/tropomyosin

Andrew H. CRIDDLE, Michael A. GEEVES* and Tracy JEFFRIES
Department of Biochemistry, Medical School, University of Bristol, Bristol BS8 1TD, U.K.

1. A pyrene label attached to Cys-374 of actin has been shown to be a useful probe for monitoring the interaction of actin with myosin subfragments [Kouyama & Mihashi (1981) Eur. J. Biochem. **114**, 33–38]. We report that the presence of this label decreases the affinity of actin for myosin subfragment 1 by less than a factor of 2. The rate of actin binding is unaffected by the label and the dissociation rate is increased by up to a factor of 2. 2. Both the rate of actin binding to, and the rate of actin dissociation from, heavy meromyosin show two phases when monitored by pyrene fluorescence. 3. Thin filaments reconstituted from pyrene-labelled actin show a 5% increase in pyrene fluorescence on binding Ca^{2+}.

INTRODUCTION

The interaction of actin with myosin and the associated thin-filament proteins is central to the molecular events leading to muscle contraction. Studies of the nature of the interaction between actin and these other muscle proteins are limited by the sensitivity of the techniques used to monitor the interaction. In general the techniques used (light-scattering, protein fluorescence, ultracentrifugation) are most accurate at protein concentrations above 0.5 μM. As the affinity of actin for myosin subfragment 1 (S1) is of the order of 1×10^7 M^{-1} and for heavy meromyosin (HMM) is 1×10^{10} M^{-1}, the use of these techniques to measure affinities is difficult. In addition, these techniques give limited information about changes in the interactions between actin and other proteins in response to nucleotide and Ca^{2+} binding. The use of fluorescently labelled actin is one approach that may yield information on these complex interactions by increasing the sensitivity of the measurements and by reporting conformational changes of the proteins. Labelled actin has been used previously, but the fluorophores have not significantly increased the sensitivity of the measurements (Porter & Weber, 1979; Marston, 1980, 1982).

Kouyama & Mihashi (1981) reported that actin could be labelled specifically at Cys-374 by *N*-(1-pyrenyl) iodoacetamide. This highly fluorescent group, which can label actin to more than 90%, responds to S1 or HMM binding by a 70% quenching of the pyrene fluorescence. The high fluorescence of the pyrene group coupled with the very large quench produced by the binding of S1 allows the interaction of actin with S1 to be studied at much lower protein concentrations than has been possible previously. We report here the use of this modified actin to study the equilibrium and dynamics of the interaction of actin with S1. In addition, preliminary studies demonstrate the potential of this label for studying the complex interactions of actin with troponin (Tn), tropomyosin (Tm) and both actin-binding sites of HMM.

MATERIALS AND METHODS

S1 was prepared by chymotryptic digestion of rabbit muscle myosin essentially as described by Weeds & Taylor (1975). For most experiments the A1 isoenzyme was used, but in some cases a mixture of the two enzymes was also used. We have no evidence of any differences between the isoenzymes in the way in which they interact with actin at the ionic strengths used in the present study, confirming previous studies (Wagner *et al.*, 1979; Chalovich *et al.*, 1984). Concentrations are quoted on the basis of M_r 115000 and $A^{1\%}_{280,\,1cm}$ 7.7 (Margossian & Lowey, 1982). F-actin was prepared from an acetone-dried powder as described by Lehrer & Kerwar (1972); concentrations were determined from $A^{1\%}_{280,\,1cm} = 11.08$ and M_r 42000 (West *et al.*, 1967).

Pyrene-labelled actin was prepared essentially by the method of Cooper *et al.* (1983). F-actin was incubated at room temperature, in the dark, for 16 h with a 1.5 molar excess of *N*-(1-pyrenyl)iodoacetamide (Molecular Probes, Junction City, OR, U.S.A.). The protein was at a concentration of 1 $mg \cdot cm^{-3}$ and pyrene was introduced as a 4 mM solution in dimethylformamide. Unchanged label and denatured protein were removed by centrifugation at 2000 *g* for 1 h and labelled protein was collected by centrifugation at 70000 *g* for 3 h. Labelled actin was assayed as G-actin. The concentration of label present was calculated from $A_{280} = 2.2 \times 10^4$ $M^{-1} \cdot cm^{-1}$ (Kouyama & Mihashi, 1981). Protein concentrations were determined by using the Bradford (1976) method; this enabled the determination of the absorption coefficient at 280 nm (2.33×10^4 $M^{-1} \cdot cm^{-1}$) for the pyrene group, allowing a routine spectrophotometric assay of the protein and label concentrations. Protein preparations normally gave 50–70% yield with more than 90% labelling.

Abbreviations used: S1, myosin subfragment 1; HMM, heavy meromyosin; pyr-A (in equations), pyr-actin, pyrene-labelled actin; Tn, troponin; Tm, tropomyosin; IAEDANS, *N*-iodoacetyl-*N'*-(5-sulpho-1-naphthyl)ethylenediamine.

* To whom correspondence and reprint requests should be addressed.

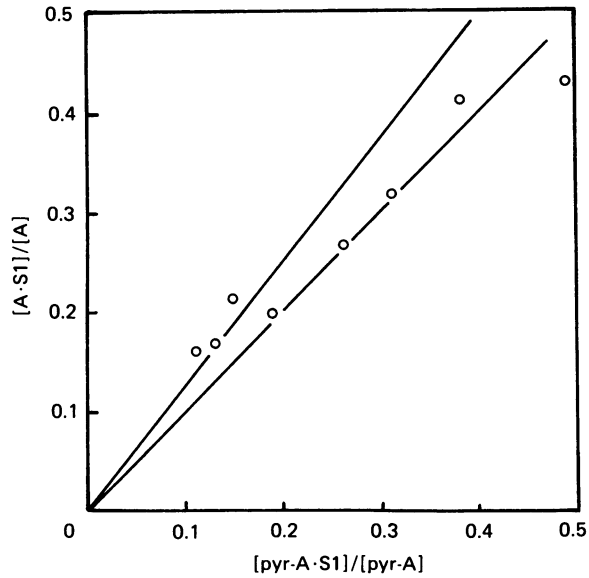

Fig. 1. Relative affinity of pyr-actin and actin (A) for S1

Conditions: 0.1 M-KCl, 5 mM-$MgCl_2$, 20 mM-imidazole (pH 7 at 23 °C), 10 μM-pyr-actin, 5 μM-S1 and 0–20 μM-actin. The data were plotted according to eqn. (1) and the straight lines represent $K/K' = 1.0$ and 1.25.

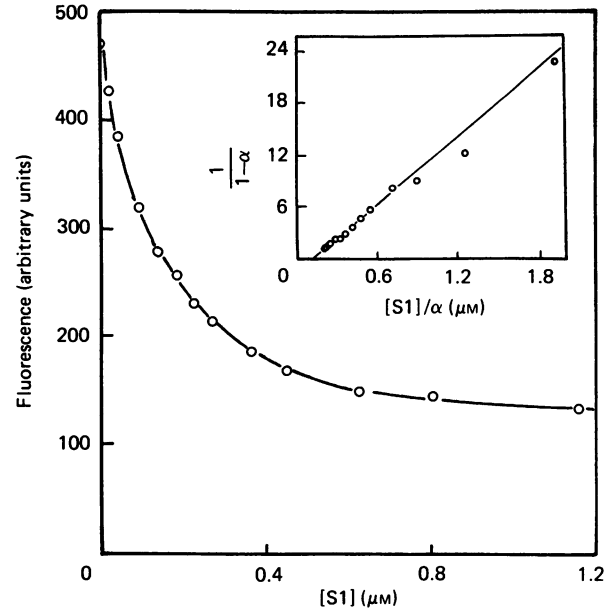

Fig. 2. Change in fluorescence of a solution of 0.2 μM-pyr-actin as the concentration of S1 is increased from 0 to 1.2 μM

The buffer was 0.1 M-KCl/5 mM-$MgCl_2$/20 mM-imidazole, pH 7 at 23 °C. The inset shows data plotted according to eqn. (2).

Fluorescence

Fluorescence spectra were measured on a SLM fluorimeter. The titration of pyr-actin with S1 and the displacement of labelled actin from pyr-acto·S1 by successive additions of native actin were performed on a Farrand fluorimeter.

Transient kinetics

Transient kinetic experiments were performed on a Hi-Tech (Salisbury, Wilts., U.K.) stopped-flow fluorimeter. Light-scattering was monitored by illumination at 365 nm with a mercury lamp and a Bausch and Lomb monochromator and detected at 90° to the incidence light. Pyrene fluorescence was monitored by using the same optical arrangement, except that the exciting light was passed through a Schott UG11 glass filter before entering the sample cell and the emitted light was detected through a Schott KV 393 glass filter. The temperature of the stopped-flow machine was maintained within 0.1 °C during the course of the experiment.

Kinetic data were collected on a Data Lab. DL905 or a Nicolet 3091 transient recorder and then transferred to a ITT 2020 microcomputer for storage and subsequent analysis. The data were analysed by a non-linear least-squares fitting routine as described by Edsall & Gutfreund (1983).

RESULTS AND DISCUSSION

The fluorescence spectra of pyr-actin, as reported by Kouyama & Mihashi (1981), show two peaks in both the excitation and emission spectrum which are quenched on S1 binding. Those authors also reported that the quenching produced by either S1 or HMM is linearly related to the degree of saturation of the actin with myosin subfragment. For most of the experiments reported here, the fluorescence was monitored by exciting at 368 nm and measuring emission at 407 nm. Under these conditions the quenching produced by S1 was 68–70%. The fluorescence of pyr-actin is also sensitive to ionic strength, a quenching of 50% occurring on increasing the KCl concentration from 0.01 to 0.5 M. The additional quenching produced on S1 binding remains, however, at 60–70%.

The relative affinity of actin and pyr-actin for S1

It is important to establish with any study using a modified protein the extent to which the modification of the protein affects the parameters of interest. The relative affinity of S1 for actin and pyr-actin was measured by monitoring the change in the fluorescence of a solution of 10 μM-pyr-actin and 5 μM-S1 as the concentration of native actin was increased from 0 to 20 μM. The pyrene fluorescence increased as the native actin displaced pyr-actin from the pyr-acto·S1 complex. The fluorescence signal allowed the concentration of bound and free pyr-actin to be calculated and, provided that the concentration of free S1 is negligible, then the concentration of bound and free native actin can be obtained. If the association constants for S1 to the two actin populations (A, pyr-A) are given by

$$K_a = \frac{[A\cdot S1]}{[A]\,[S1]}, \qquad K'_a = \frac{[\text{pyr-A}\cdot S1]}{[\text{pyr-A}]\,[S1]}$$

Then:

$$\frac{[A\cdot S1]}{[A]} = \frac{K_a}{K'_a}\,\frac{[\text{pyr-A}\cdot S1]}{[\text{pyr-A}]} \qquad (1)$$

and the data can be plotted as in Fig. 1 to produce a straight line with a gradient of the ratio of the two association constants.

Table 1. Rates and equilibrium constants for the interaction of pyr-actin with S1

Conditions	Parameter...	K_a (M^{-1})	K_0k_{+1} ($M^{-1}\cdot s^{-1}$)	k_{-1} (s^{-1})	K_0k_{+1}/k_{-1}
0.1 M-KCl, 20 °C, pH 7		9.1×10^6	1.24×10^6	0.22	5.6×10^6
0.5 M-KCl, 20 °C, pH 7		2.7×10^5	1.6×10^5	0.90	1.8×10^5
0.1 M-KCl, 3 °C, pH 7		3.0×10^5	—	0.10	—

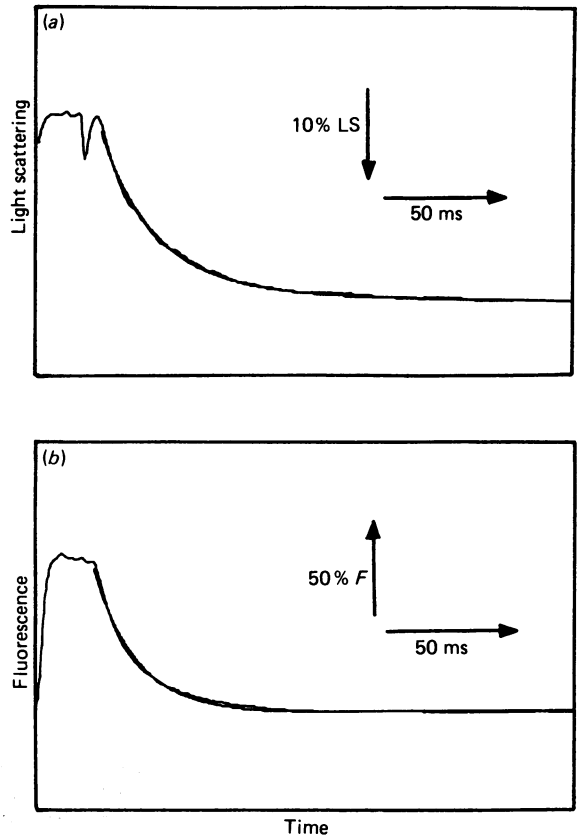

Fig. 3. Rate of S1 association with actin and pyr-actin

The traces represent the average results for four successive reactions in the stopped-flow machine and the computer-drawn best-fit exponental is superimposed. The buffer was 0.1 M-KCl/5 mM-$MgCl_2$/20 mM-imidazole, pH 7 at 20 °C. (*a*) Change in light-scattering (LS) on mixing 2 μM-actin and 40 μM-S1 ($k = 49\ s^{-1}$); (*b*) change in fluorescence (*F*) on mixing 2 μM-pyr-actin and 40 μM-S1 ($k = 54\ s^{-1}$).

The method is not highly accurate, since it depends on knowing the concentration of the three proteins accurately; any contaminating protein or inactive protein will distort the result. The data do, however, suggest that there is no major change in the affinity of actin for S1 by the modification and that the affinity for S1 is decreased by a maximum of 50% by the presence of the label.

Titration of pyr-actin with S1

The high sensitivity of the pyrene fluorescence allowed the association constant of S1 for pyr-actin to be measured directly by fluorescence titration. Fig. 2 shows the change in the fluorescence of 0.2 μM-pyr-actin as S1 is titrated into the cuvette. The data were analysed by using the following equation:

$$\frac{1}{1-\alpha} = \left(\frac{\alpha}{[S1]} - [\text{pyr-A}]\right) K_a \qquad (2)$$

where K_a is the association constant, α the fraction of pyr-actin bound to S1 and [pyr-A] and [S1] refer to the total concentrations of the two proteins.

This analysis provides a value of $9.1\times10^6\ M^{-1}$ for the association constant. Increasing the KCl concentration of the buffer to 0.5 M decreases the association constant to $2.7\times10^5\ M^{-1}$, and a similar decrease in affinity is produced by a 20 °C lowering of the temperature at 0.1 M-KCl (Table 1).

Stopped-flow studies of acto·S1 association and dissociation

The association reaction between actin and myosin subfragments has been studied by several workers by using the increase in light-scattering resulting from the formation of the actomyosin complex (Finlayson *et al.*, 1969; White & Taylor, 1976). We report here the use of pyr-actin to monitor this reaction.

The results of a typical reaction observed on mixing pyr-actin with an excess of S1 are shown in Fig. 3(*b*). The same reaction studied by using native actin and monitoring 90° light-scattering are shown in Fig. 3(*a*). The signal change for light-scattering is in the opposite direction to that observed for pyrene fluorescence; by using our optical set-up there appears to be no interference from light-scattering changes when monitoring pyr-actin fluorescence.

The light-scattering changes that theoretically occur in the case of the pyr-actin cannot be successfully monitored, owing to the absorbance changes in pyrene occurring in the range 330–400 nm. Plots of the concentration-dependence of the observed rates for both native and pyr-actin are shown in Fig. 4. The plots are linear over the observed concentration range and the rates observed for native and pyr-actin are indistinguishable. The linear relationship between the observed rates and S1 concentration suggest that a single-step binding model will fit the data adequately. However, the large temperature-dependence of the second-order rate constants [Konrad & Goody, 1982; Marston, 1982; the following paper (Coates *et al.*, 1985)] suggest that at least two steps are involved:

$$A + S \underset{}{\overset{K_0}{\rightleftharpoons}} A\cdot S_0 \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} A\cdot S_1$$

where $A\cdot S_0$ is the collision complex and the second step represents an isomerization of the binary complex to give the stable rigor-type bond. For this model, assuming that the formation of the collision complex is a fast

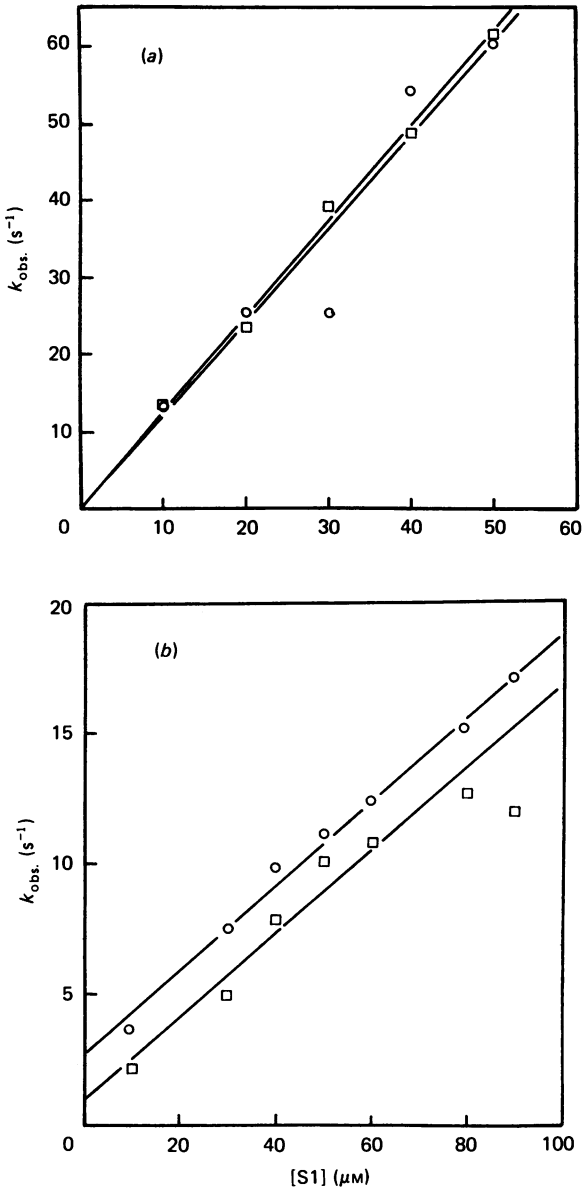

Fig. 4. Dependence of the observed rate of association of actin and S1 on S1 concentration

○, Pyr-actin; □, actin. (*a*) Conditions were as for Fig. 3. Best-fit lines: actin gradient, 1.22×10^6 M$^{-1}\cdot$s^{-1}; pyr-actin gradient, 1.24×10^6 M$^{-1}\cdot$s^{-1}. (*b*) As (*a*) but KCl = 0.5 M. Best-fit lines: actin gradient, 1.6×10^5 M$^{-1}\cdot$s^{-1}, intercept, 0.9 s^{-1}; pyr-actin gradient, 1.6×10^5 M$^{-1}\cdot$s^{-1}; intercept, 2.7 s^{-1}.

equilibrium step complete within the mixing time of the apparatus, then:

$$k_{obs.} = \frac{K_0 k_{+1}[S]}{K_0[S]+1} + k_{-1}$$

which is linear when $K_0[S] < 1$ and the gradient and intercept of Fig. 4 represent $K_0 k_{+1}$ and k_{-1} respectively. At 0.5 M-KCl the rates observed for the two actins differ (Fig. 4*b*), and this can be attributed to a change in k_{-1} (0.9 s^{-1} for native actin and 2.7 s^{-1} for pyr-actin). The intercepts in Fig. 4(*a*) are not defined sufficiently accurately to determine whether k_{-1} changes in a similar manner at 0.1 M-KCl, but any change in K_a must be reflected in a change in k_{-1}.

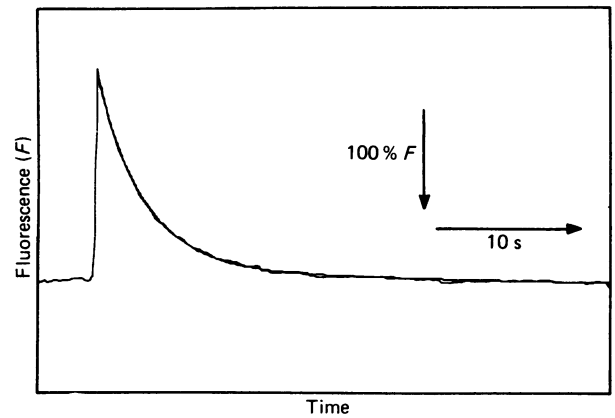

Fig. 5. Stopped-flow displacement of pyr-actin from pyr-acto·S1 by the addition of excess native actin

Conditions: 1.1 μM-pyr-A, 1.2 μM-S1, 5 μM-actin; other conditions were as for Fig. 3. $k_{obs.} = 0.22$ s^{-1}.

The rate of release of S1 from pyr-actin was measured directly by displacing the pyr-actin with an excess of native actin. This was similar to the experiment above (Fig. 1), but in this case the time course of the fluorescence change was monitored in a stopped-flow apparatus. Such an experiment is shown in Fig. 5, where 1.1 μM-pyr-actin was displaced from a small excess of S1 by the addition of a 5-fold excess of native actin. The observed rate was 0.22 s^{-1} and corresponds to k_{-1}. The observed rate was unaffected by increasing the concentration of native actin used in the experiment. At 0.5 M-KCl the rate was 0.9 s^{-1} and at 2 °C in 0.1 M-KCl it was 0.1 s^{-1}. The dissociation rate measured here is a factor of 3 lower than the value obtained from the intercept of Fig. 5(*b*). Dissociation rates measured from intercepts are inaccurate when the observed rates are much greater than the value of the intercept, as is the case here; therefore the values obtained by direct displacement are preferred. However, this discrepancy remains surprisingly large.

The value of the rate constant of the association reaction obtained from both light-scattering and fluorescence measurements are in good agreement with each other and with those obtained by other workers under similar conditions (White & Taylor, 1976; Konrad, 1981; Marston, 1982; Geeves & Gutfreund, 1982). The dissociation rate constant is more difficult to approach experimentally, and few direct measurements of this rate constant have been made. The approach used here is experimentally difficult because of the problem of mixing two viscous actin solutions and because the observed optical signal can be affected by slow aggregation phenomena. Aggregation problems varied from preparation to preparation and were considerably worse for older protein preparations. We excluded any experimental data which showed any deviation from a single exponential and any slow drift of the baseline.

Marston (1982) measured the dissociation rate directly by using the same approach, but by using actin labelled with IAEDANS. The rates that he obtained were only one-tenth of those which we observed. This difference is too large to be accounted for by the differences between

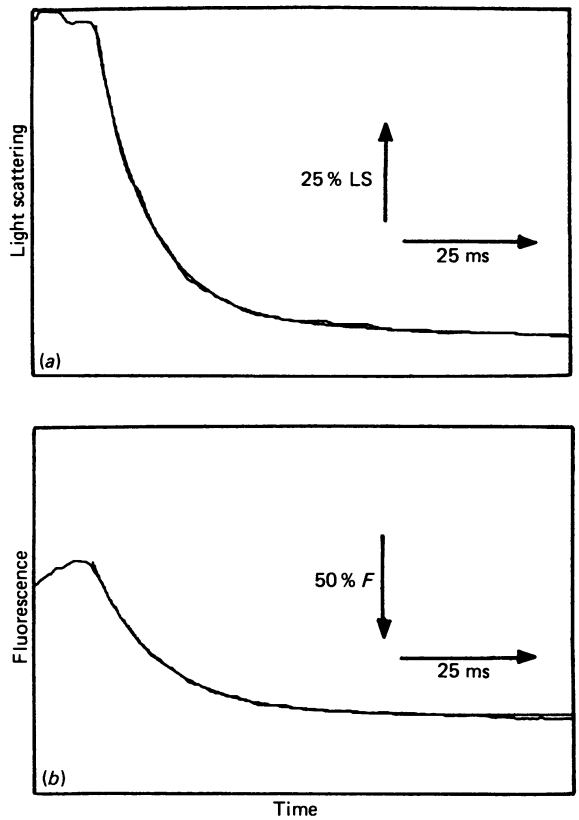

Fig. 6. ATP-induced dissociation of acto·S1 and pyr-acto·S1 at 0.5 °C

(*a*) Change in light-scattering (LS) on mixing 2 μM-acto·S1 and 250 μM-ATP; $k_{obs.} = 108\ s^{-1}$. (*b*) Change in fluorescence (F) on mixing 2 μM-pyr-acto·S1 and 250 μM-ATP; $k_{obs.} = 80\ s^{-1}$. All other conditions were as for Fig. 4.

the temperatures and ionic strengths used in the two experiments [Marston's (1982) conditions: 60 mM-KCl, 25 °C]. We have shown that the use of pyrene label increases the dissociation rate by a factor of 2 at 0.5 M-KCl and that a weakening of the affinity of actin for S1 at lower ionic strength is compatible with a similar decrease in the dissociation rate. The effect of the IAEDANS label on actin S1 interaction is less well documented, but Marston (1982) has shown that the association rate constant (K_0k_{+1}) is unaffected. A small increase in the affinity of actin and S1 caused by this label decreasing the dissociation rate would be compatible with the results reported here. The ratio of the rate constants obtained from these experiments can be compared with the association constants obtained by direct titration. This is shown in Table 1, and the values of K_0k_{+1}/k_{-1} are in good agreement with those of K_a.

The overall binding constant of actin for S1 has been measured many times by using a variety of techniques; sedimentation (Margossian & Lowey, 1978; Marston & Weber, 1975), fluorescence depolarization (Highsmith *et al.*, 1976), ratio of rate constants (White & Taylor, 1976; Marston, 1982), and detailed balance of nucleotide and actin binding of S1 (Konrad & Goody, 1982; Inoue & Tonomura, 1980). The value of the binding constant obtained depends on the temperature and ionic strength at which the measurements were made, but this does not account for all the variability observed. A value between 10^7 and $10^8\ M^{-1}$ would be compatible with most of the results quoted above at 0.1 M-KCl and 20 °C, and the results obtained here lie within this range.

Yasui *et al.* (1984) reported the use of the same label to study the interaction of actin and S1, and their results differ in several respects from the results reported here. The primary discrepancy is that they observe a rate constant of association (K_0k_{+1}) of pyr-actin with S1 that is ten times the rates that we observed, although the dissociation rate (k_{-1}) they measured was in good agreement with our results. Their second-order rate constants were obtained from plots of observed rate against [S1] as shown in Fig. 4, except that in their case the range of S1 concentrations used were 0.3–0.9 μM with actin at 0.1 μM compared with the 10–50 μM-S1 and 2 μM-actin shown here. We used the higher concentrations of proteins to prevent any artefacts arising from the presence of depolymerized actin. Cooper *et al.* (1983) have shown that the critical concentration of actin polymerization under these conditions is 0.6 μM. The rates of the association reaction that we measured here are in good agreement with those obtained using pyr-actin in pressure–relaxation experiments (Coates *et al.*, 1985).

ATP-induced dissociation of acto·S1

One further way of characterizing the effect of the pyrene label on the interaction of actin and S1 is to examine the rate of the ATP induced dissociation of acto.S1. The observed rate of the dissociation has been shown to be linearly dependent on concentration (Taylor & Weeds, 1976; White & Taylor, 1976). At 0.5 °C, Millar & Geeves (1983) showed that the relationship became hyperbolic and they proposed the following model to account for this observation:

$$A\cdot S+ATP \overset{1}{\rightleftharpoons} A\cdot S\cdot ATP \overset{2}{\rightleftharpoons} A\cdot S'\cdot ATP \overset{3}{\rightarrow} A+S\cdot ATP$$

where step 1 and step 3 are much faster than step 2, then:

$$k_{obs.} = \frac{K_1k_{+2}[ATP]}{K_1[ATP]+1}.$$

We found that, at 20 °C, the observed rate of the dissociation was linearly dependent on [ATP], as has been shown by others, and that the rates observed with pyr-actin were indistinguishable from those with native actin. At 0.5 °C, however, when we repeated the experiment of Millar & Geeves (1983), the labelled actin did show a significant difference from the native actin. The results are shown in Figs. 6 and 7. At low [ATP], where the rates are linearly dependent on [ATP], the rates appear to be identical. At high [ATP] the maximum rate observed ($= k_{+2}+k_{-2}$) for pyr-actin is approximately half that of native actin, and complete analysis of the curves in Fig. 7 show that this is compensated for at low ATP by an increase in K_1.

Interaction of pyr-actin with HMM

HMM, the myosin subfragment with two ATP- and two actin-binding sites located in two separate protein domains, or heads, is believed to interact with actin in a very similar way to S1, i.e. the sites act independently. The affinity of actin for HMM is $10^{10}\ M^{-1}$ (Greene &

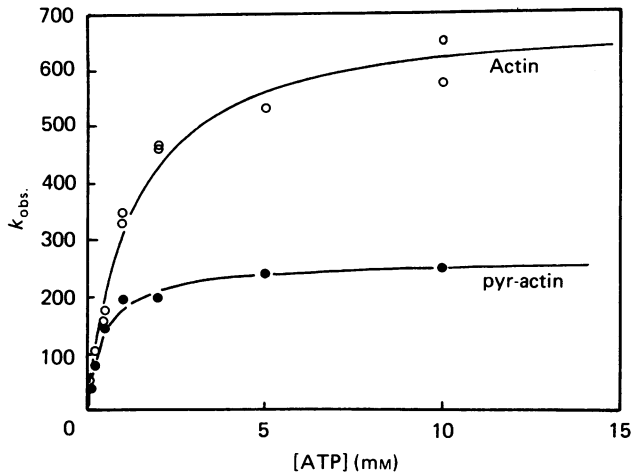

Fig. 7. Dependence of the observed rate of dissociation on ATP concentration at 0.5 °C

○, Actin: $K_1 = 0.79 \times 10^3$ M^{-1}; $k_{+2} = 708$ s^{-1}; ●, pyr-actin: $K_1 = 1.96 \times 10^3$ M^{-1}; $k_{+2} = 266$ s^{-1}.

Eisenberg, 1980) and is too tight to be measured by the titration method using pyr-actin.

The kinetics of HMM binding to actin has been studied previously by monitoring changes in light-scattering and has revealed a reaction very similar to that seen with S1 (White & Taylor, 1976). The measurement of the association reaction by light-scattering only reveals information about the first head that binds; the second head, which is believed to bind to the adjacent actin monomer on the actin filament, produces no further change in the light-scattering signal. It is not clear whether the second binding step is a rapid first-order step as might be expected from this model or if steric factors slow the reaction down significantly. The use of a fluorescence probe on actin will report the binding of both HMM heads to actin. Kouyama & Mihashi (1981) reported that both S1 and HMM produce the same quenching of pyr-actin at a molar ratio of 1:1 and 1:2 respectively, suggesting that the second head of HMM quenches the fluorescence of pyrene in the same way as S1. Stopped-flow traces of HMM binding to actin and pyr-actin are shown in Fig. 8. The light-scattering signal from native actin fits a single exponential, as has been reported by White & Taylor (1976). The reaction with pyr-actin, however, shows two distinct phases. The fast phase is similar in rate to that observed in light-scattering; the slow phase comprises one-third of the total amplitude and has a rate one-sixth of the fast phase in this example. Both the ratio of rates and the ratio of amplitudes vary with protein concentration. A biphasic change in fluorescence is also observed if pyr-actin is displaced from the complex with HMM by the addition of excess native actin as shown in Fig. 9.

The biphasic nature of these reactions could be a product of heterogeneity of the proteins, but as monophasic reactions are seen with S1, any heterogeneity can only reside in the HMM. In the association reaction a 5-fold excess of HMM is mixed with pyr-actin. In order for heterogeneous HMM to produce the observed results, a fraction of the HMM must bind much more slowly to pyr-actin yet compete with the fast-binding fraction. This

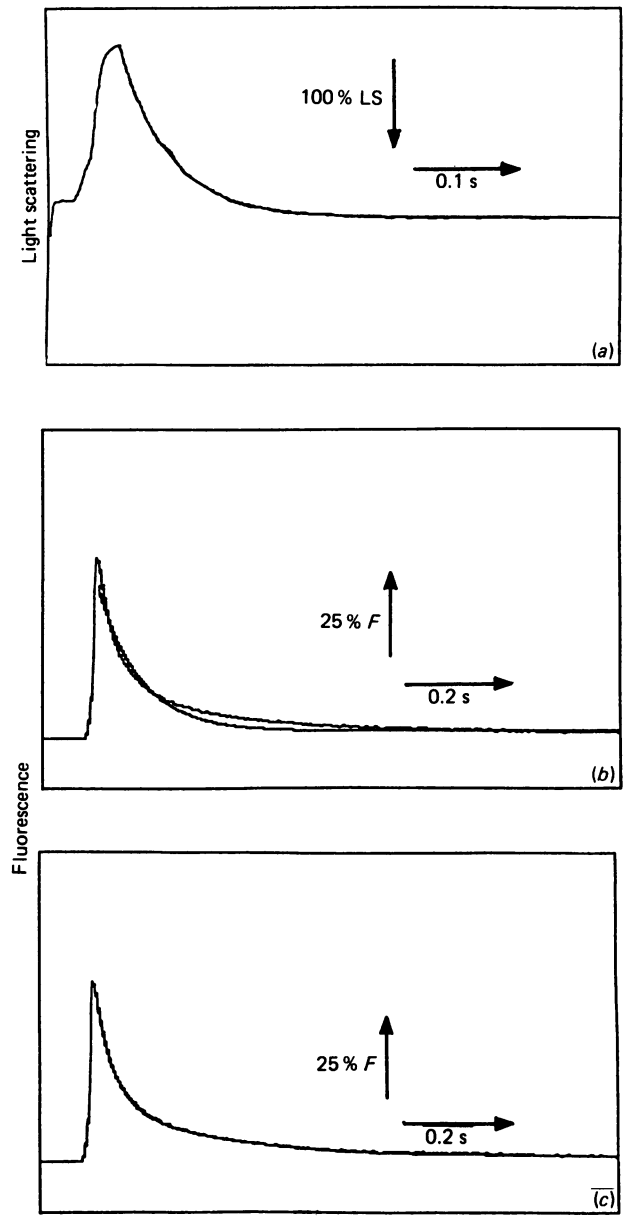

Fig. 8. Rate of association of HMM and actin

(*a*) Change in light-scattering (LS) on mixing 2 μM-actin and 10 μM-HMM ($k = 22.7$ s^{-1}). (*b*) Change in fluorescence (F) on mixing 2 μM-pyr-actin and 10 μM-HMM ($k = 13.8$ s^{-1}). (*c*) As (*b*), but the data fitted to a double exponential [$k_1 = 33.0$ s^{-1}; $k_2 = 5.5$ s^{-1}; ratio of amplitudes (1/2) = 2:1].

heterogeneity would also be apparent in the data with unlabelled actin. We conclude, therefore, that the biphasic reactions are not due to heterogeneity in the proteins.

Complete kinetic analysis of these reactions is complex and beyond the scope of the current work, but we believe that complete solution must allow for a fraction of the HMM to interact with two actin filaments as has been shown to occur by electron microscopy in both muscle fibres and in acto-HMM solutions (Offer & Elliot, 1978; Trinick & Offer, 1979).

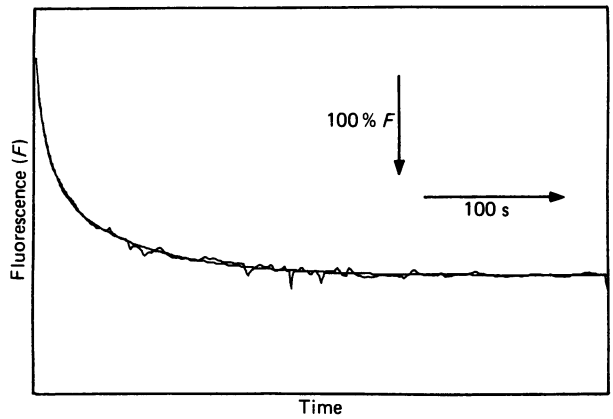

Fig. 9. Stopped-flow displacement of pyr-actin from pyr-acto·HMM by the addition of excess native actin

Conditions: 0.49 μM-HMM, 0.77 μM-pyr-actin. Double-exponential fit gave $k_1 = 0.13\ s^{-1}$; $k_2 = 0.016\ s^{-1}$; ratio of amplitudes (A1/A2) = 1.09. All other conditions were as for Fig. 3.

Interaction of pyr-actin with Tn and Tm

We have reconstituted rabbit skeletal-muscle thin filaments with the labelled actin by using the method of Murray (1982). These filaments activate the S1 ATPase 20-fold in the presence of Ca^{2+} (0.03 M-KCl, pH 7.5, 20 °C, 5 μM-pyr-A) and this activation is > 95% inhibited in the absence of Ca^{2+}. This is the same level of inhibition as we observe with unlabelled thin filaments. The fluorescence signal of the thin filaments is identical with that of pyr-actin and shows the same degree of quenching on binding S1. In addition, the thin filaments show a small (5%) increase in fluorescence in response to Ca^{2+} binding. We have used this signal to monitor the rate of Ca^{2+} release from thin filaments. In these experiments thin filaments in the presence of 0.5 mM-Ca^{2+} were mixed with 5–20 mM-EGTA, and we observed a decrease in fluorescence with a rate of 20 s^{-1} independent of the EGTA concentration. Ca^{2+} binding and release from Tn C has been measured in a similar approach by using Tn C labelled with dansylaziridine (5-dimethylamino-naphthalene-1-sulphonylaziridine) (Johnson *et al.*, 1979). The rate at which Ca^{2+} was released from Tn C was measured as 300–350 s^{-1} and estimated as 20 s^{-1} in whole Tn. The rate measured here is in good agreement with this estimate. The small fluorescence change observed here does not allow us to distinguish between a model in which actin monomers adjacent to Tn C change their fluorescence in response to Ca^{2+} binding or a model in which all the actin monomers respond to the Ca^{2+}-induced conformational change in Tm.

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