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Fluorescence Resonance Energy Transfer within the Complex Formed by Actin and Myosin Subfragment 1. Comparison between Weakly and Strongly Attached States†

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ABSTRACT: Fluorescence resonance energy transfer measurements have been made between Cys-374 on actin and Cys-177 on the alkali light chain of myosin subfragment 1 (S1) using several pairs of donor–acceptor chromophores. The labeled light chain was exchanged into subfragment 1 and the resulting fluorescently labeled subfragment 1 isolated by ion-exchange chromatography on SP-Trisacryl. The efficiency of energy transfer was measured by steady-state fluorescence in a strong binding complex of acto–S1 and found to represent a spatial separation between the two probes of 5.6–6.3 nm. The same measurements were then made with weak binding acto–S1 complexes generated in two ways. First, actin was complexed with *p*-phenylenedimaleimide–S1, a stable analogue of S1–adenosine 5′-triphosphate (ATP), obtained by cross-linking the SH_1 and SH_2 heavy-chain thiols of subfragment 1 [Greene, L. E., Chalovich, J. M., & Eisenberg, E. (1986) *Biochemistry 25*, 704–709]. Large increases in transfer efficiency indicated that the two probes had moved closer together by some 3 nm. Second, weak binding complexes were formed between subfragment 1 and actin in the presence of the regulatory proteins troponin and tropomyosin, the absence of calcium, and the presence of ATP [Chalovich, J. M., & Eisenberg, E. (1982) *J. Biol. Chem. 257*, 2432–2437]. The measured efficiency of energy transfer again indicated that the distance between the two labeled sites had moved closer by about 3 nm. These data support the idea that there is a considerable difference in the structure of the acto–S1 complex between the weakly and strongly bound states.

It is now generally accepted that muscle contraction and the motility of many nonmuscle cells result from the relative sliding motion of myosin and actin filaments. These processes are directly linked to adenosine 5′-triphosphate (ATP)[1] hydrolysis and involve the head of the myosin molecule (cross bridge) interacting in cycles of detachment and reattachment with the actin filaments. Currently held theories as to the nature of the force-generating step suggest that during each cycle of ATP hydrolysis the cross bridges alternate between at least two main conformations (Eisenberg & Greene, 1980). The binding of ATP to the myosin induces a cross-bridge structure which binds weakly to actin, the free and actin-bound cross bridges being in rapid equilibrium. Following hydrolysis of ATP, the release of inorganic phosphate transforms the cross bridge to the strong binding conformation. This strong binding or "rigor" complex is easily studied because it is stable and long-lived. In contrast, however, the weakly bound states are

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[1] Abbreviations: ADP, adenosine 5′-diphosphate; ATP, adenosine 5′-triphosphate; Bicine, *N,N*-bis(2-hydroxyethyl)glycine; 1,5-BrAEDANS, 5-[[2-[(bromoacetyl)amino]ethyl]amino]naphthalene-1-sulfonic acid; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; 5-IAF, 5-(iodoacetamido)fluorescein; IANBD, 4-[(iodoacetoxy)-ethylmethylamino]-7-nitro-2,1,3-benzoxadiazole; 5-IAS, 5-(iodoacetamido)salicyclic acid; MOPS, 4-morphinepropanesulfonic acid; pPDM, *p*-phenylenedimaleimide; PMSF, phenylmethanesulfonyl fluoride; S1, myosin subfragment 1; S1(A1) and S1(A2), subfragment 1 containing alkali 1 (A1) or alkali 2 (A2) light chains, respectively; TEA, triethanolamine; Tn–TM, troponin–tropomyosin complex; FRET, fluorescence resonance energy transfer; TPCK, *N*-tosyl-L-phenylalanine chloromethyl ketone; HPLC, high-performance liquid chromatography; kDa, kilodalton(s), AS, acetamidosalicylic acid; AF, acetamidofluorescein.

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short-lived, and in order to evaluate their structure, attempts must be made to slow down or arrest the enzymic cycle at stages which normally occur only transiently.

Recently we described fluorescence resonance energy transfer (FRET) experiments made between a donor fluorophore, 1,5-BrAEDANS, located on Cys-177 of the A1 light chain of S1 and an acceptor fluorophore, 5-IAF, sited on Cys-374 of actin (Bhandari et al., 1985). In an attempt to provide a stable S1·ATP analogue, we cross-linked the two essential thiol groups on the S1 heavy chain (SH_1 and SH_2) with pPDM, trapping nucleotide at the active site (Wells & Yount, 1985). Extensive studies by Greene et al. (1986) have shown that pPDM·S1–nucleotide binds to actin with a similar affinity to S1·ATP and with essentially the same binding characteristics. When FRET measurements were made with the strong binding acto–S1 complex, the spatial separation of the probes was about 6 nm. When, however, the same measurements were made with the weak binding S1·ATP analogue (pPDM·S1), the two fluorophores were found to have moved closer by about 3 nm. This result provided direct experimental evidence for the existence of two different acto–S1 conformations representing the pre- and postpower stroke states.

To support these observations and to enable more precise limits to be placed on the magnitude of the change between the weak and strong binding states, experiments are described here using different donor–acceptor pairs. In addition, these studies have also been extended using another weak binding acto–S1 complex which does not require any modification of the heavy-chain sulfhydryl residues on S1. Chalovich and Eisenberg (1982) have shown that in the presence of ATP the affinity of S1 for regulated actin is relatively calcium insensitive. This is in sharp contrast to the effect of calcium on the ATPase rate, which in the absence of calcium is decreased by about 95% when compared to the rate in the presence of calcium. While these observations have profound implications regarding the mechanism of the regulatory process in skeletal muscle contraction, they also suggest that, since regulated acto–S1 turns over ATP very slowly in the absence of calcium, the weak binding complex present will be relatively long-lived. Exploiting this property, we have extended our FRET experiments to include studies with regulated actin and S1 in the absence of calcium ions and the presence of ATP to obtain a weak binding complex and in the absence of ATP to obtain the strong binding complex. As with our studies using acto–pPDM·S1, we find that in the weak binding form fluorescent probes located at Cys-374 on actin and Cys-177 on the A1 light chain in S1 move closer together by some 3 nm compared to their separation in the strong binding complex.

Materials and Methods

Chemicals. Fluorescent probes 5-IAS, 1,5-BrAEDANS, IANBD, and 5-IAF were purchased from Molecular Probes (Eugene, OR). [U-^{14}C]ADP was from Amersham International (Amersham, U.K.). ATP, ADP, PMSF, MOPS, Bicine, *N*-acetylcysteine, DTT, TPCK-treated trypsin, and α-chymotrypsin were all purchased from Sigma Chemical Co. Ltd. (Poole, Dorset, U.K.). Sephadex G-25 and Sephadex DEAE A-25 were from Pharmacia Ltd. (Bucks, U.K.) while SP-Trisacryl was purchased from LKB (Surrey, U.K.). "Aristar" grades of guanidinium chloride and ammonium chloride together with enzyme-grade ammonium sulfate were obtained from BDH Chemicals Ltd. (Dorset, U.K.). pPDM from Aldrich Chemicals (Dorset, U.K.) was further purified by sublimation.

Preparation of Proteins. Fast-twitch muscle myosin was prepared from the *longissimus dorsi* muscle of New Zealand white rabbits. The method used was essentially that of Trayer and Perry (1966) except that 0.6 M KCl, pH 6.8, was used as the extraction medium. S1 was prepared by chymotryptic digestion of myosin (Weeds & Taylor, 1975) except that 2 mM PMSF was used to terminate the reaction. The two light-chain isoenzymes of S1 were separated by ion-exchange chromatography on SP-Trisacryl in 10 mM MOPS and 0.5 mM DTT, pH 7.0, using a linear gradient from 0 to 0.2 M NaCl. Two well-separated protein peaks were obtained; the one peak eluting at the lower ionic strength contained pure S1(A2) whereas the other peak contained pure S1(A1). Light chains were extracted from myosin by the urea method and fractionated on DEAE-Sephadex as described by Henry et al. (1985).

Rabbit skeletal muscle actin was prepared from muscle acetone powder (Barany et al., 1957) by the method of Spudich and Watt (1971).

A complex of troponin and tropomyosin was prepared according to the procedure of Eisenberg and Kielley (1974).

Proteins were concentrated and stored as described by Trayer and Trayer (1983). The concentrations of unmodified proteins were determined by absorbance using the following extinction coefficients (in milligrams per milliliter per centimeter): S1 at 280 nm, 0.8; A1 and A2 light chains at 280 nm, 0.22; G-actin at 290 nm, 0.63; Tn–TM at 278 nm, 0.4. The molecular weights used for the various proteins were as follows: S(A1), 112 000; S1(A2), 106 000; G-actin, 42 000; A1 light chain, 20 700; A2 light chain, 16 500; Tn–TM, 137 200.

The concentration of labeled proteins was estimated by a slightly modified version of the microtannin turbidity method (Mejbaum-Ketzenellenbogen & Drobryszycka, 1959) as follows. The protein solution (1 mL, containing 0–50 μg of protein) was incubated at 25 °C for 5 min. To this was added 1 mL of tannin reagent prewarmed to 25 °C (10 mL of phenol in 490 mL of 1 N HCl warmed to 80 °C, 50 g of tannic acid added and dissolved; the cooled solution was filtered before use). After incubation for 10 min at 25 °C, 1 mL of 0.2% gum arabic was added and the resulting turbidity read at 520 nm. Samples of S1, actin, or light chains of known concentrations were used as standards. This method is extremely reproducible and does not suffer from the interference problems of many other colorimetric methods of protein estimation.

Protein purity was routinely analyzed by polyacrylamide gel electrophoresis under denaturing conditions as described by Trayer and Trayer (1983). ATPase activities were also measured as described by these authors except at the lower temperature of 25 °C.

Binding Studies. The amount of complex present in samples used for energy transfer was estimated by centrifugation at 199000*g* for 20 min in a Beckman Airfuge. The concentration of free S1 was determined by fluorescence measurements or NH_4^+-EDTA ATPase activity (Chalovich & Eisenberg, 1982), and the data were analyzed as described previously (Trayer & Trayer, 1985).

Fluorescent Probes. 1,5-BrAEDANS was soluble in aqueous buffers, and a 10 mM solution in 25 mM TEA-HCl, pH 8.0, was routinely used for labeling proteins. The molar absorption coefficient at 338 nm was determined to be 4.536 $\times 10^3$ M^{-1} cm^{-1}. Reaction with *N*-acetylcysteine did not alter this value, but the absorption maximum shifted to 340 nm. 5-IAS was also soluble in 25 mM TEA-HCl, pH 8, but had no clearly defined absorption maximum until reacted with thiol adducts. After reaction with *N*-acetylcysteine, the molar absorption coefficient at 308 nm was determined to be 3.2 ×

10^3 M^{-1} cm^{-1}. IANBD was readily soluble in acetonitrile at relatively high concentrations (~100 mM). Samples could then be diluted with 25 mM TEA-HCl, pH 8, provided that the final concentration of IANBD was no greater than 0.3 mM. Reaction with *N*-acetylcysteine did not appreciably alter the molar absorption coefficient at 495 nm of 2.6×10^4 M^{-1} cm^{-1}. 5-IAF was soluble at about 3 mM in 25 mM TEA-HCl, pH 8. The molar absorption coefficient at 494 nm was determined to be 7.127×10^4 M^{-1} cm^{-1}, whether or not reacted with *N*-acetylcysteine.

Fluorescent Labeling of Actin. Freeze-dried actin was reconstituted in 5 mM TEA-HCl, 0.2 mM $CaCl_2$, 0.2 mM ATP, and 0.25 mM DTT, pH 8.0 (buffer A), and dialyzed against 200 volumes of the same buffer overnight. In most cases, G-actin (3–6 mg·mL^{-1}) was labeled for 4 h at 4 °C in the dark using either a 5-fold molar excess of IANBD, a 10-fold molar excess of 5-IAS, or a 15-fold molar excess of 1,5-BrAEDANS. Modification with 5-IAF required a 20-fold molar excess of probe for at least 18 h. Subsequently, any unreacted fluorophore was removed from the actin on Sephadex G-25 equilibrated in buffer A, followed by dialysis against the same buffer. Prior to any fluorescence measurements, the G-actin was polymerized by the addition of KCl to 50 mM and $MgCl_2$ to 2 mM. After at least 1 h at 4 °C, the F-actin was dialyzed against 200 volumes of 5 mM TEA-HCl, 10 mM KCl, 2 mM $MgCl_2$, and 0.25 mM DTT, pH 8.0, for 2 h on a rocking dialyzer to remove excess ATP.

Determination of the stoichiometry of labeling has been described elsewhere (Trayer & Trayer, 1983) and varied between 0.7 and 1.1 mol/mol of protein. Preincorporation of *N*-ethylmaleimide into actin essentially prevented subsequent labeling with fluorophore, providing evidence for single-site labeling at Cys-374 (Trayer & Trayer, 1983). This was confirmed by isolation of the C-terminal cyanogen bromide fragment as described in Trayer et al. (1987).

Fluorescent Labeling of the A1 Light Chain. Sixty-three milligrams of freeze-dried A1 light chain (3 μmol) was dissolved in 1 mL of 0.5 M TEA-HCl, 6 M guanidine hydrochloride, and 2 mM EDTA, pH 8.0. To this was added 20 mg of DTT (130 μmol) and left to reduce at room temperature overnight. The DTT was then decreased to 2 mM by passing the reduced A1 down Sephadex G-25 (1 × 28 cm) equilibrated in 0.5 M TEA-HCl, 6 M guanidine hydrochloride, 2 mM EDTA, and 2 mM DTT, pH 8.0. The light chain was then immediately reacted with the fluorescent probe. Approximately 35 mg of solid 5-IAF (68 μmol) was added to give a 20–25-fold molar excess over A1. This was left stirring in the dark at room temperature for about 20 h. BrAEDANS was also added in a 20-fold molar excess (25 mg) but was only left reacting for about 2 h at room temperature, in the dark. Excess probe and guanidine hydrochloride were immediately removed on Sephadex G-25 (2.2 × 32 cm) equilibrated in 25 mM TEA-HCl and 0.5 mM DTT, pH 8.0. Before being freeze-dried, the labeled light chains were exhaustively dialyzed against 0.1% NH_4HCO_3 containing 20 mM β-mercaptoethanol.

The level of incorporation of fluorescent probe into A1 light chain was determined as described in Trayer and Trayer (1983) and was in the range of 0.6–1.0 mol/mol of protein.

Peptide Analysis of Labeled A1 Light Chains. The specificity of labeling A1 light chains was investigated by HPLC analysis of the tryptic peptides. Freeze-dried labeled light chains (3.5 mg) were dissolved in 1 mL of 0.1 M NH_4HCO_3, pH 8.1, and digested with TPCK-treated trypsin for 3 h at 37 °C with a light-chain:trypsin ratio of 100:1 by weight. A

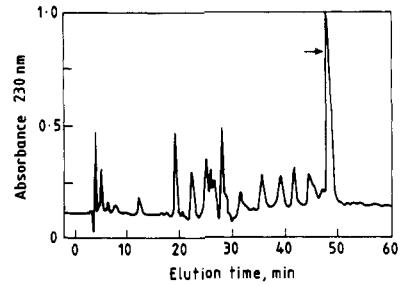

FIGURE 1: Separation of tryptic peptides of A1–AEDANS light chain by reverse-phase HPLC on a column of C8 Zorbax SP150 (250 × 4.6 mm). Twenty microliters (17 nmol) of digested A1–AEDANS was applied in 0.1% trifluoroacetic acid and eluted with a gradient of acetonitrile as described under Materials and Methods. Peptide elution was monitored at 230 nm. Flow rate 1 mL·min^{-1}. The arrow indicates the only fluorescent peptide [taken from Bhandari (1985)].

further addition of an equivalent amount of trypsin was then added and incubated at 37 °C overnight. The digest was then freeze-dried and dissolved in 1 mL of 0.1% trifluoroacetic acid. This process was repeated once more. Finally, the digest was dissolved in 0.2 mL of 1% trifluoroacetic acid injected onto a C8 Zorbax SP150 (250 × 4.6 mm) reverse-phase HPLC column (Dupont Co., Wilmington, DE) equilibrated in 0.1% trifluoroacetic acid. After being washed for 3 min, the peptides were eluted under the following conditions: from 3 to 18 min, a linear gradient of 0–15% acetonitrile in 0.1% trifluoroacetic acid was applied followed from 18 to 60 min with a linear gradient of 15–30% acetonitrile in 0.1% trifluoroacetic acid (Figure 1). The fluorescence of all the peptide peaks was then determined by monitoring the emission at 480 nm of samples excited at 360 nm. Only the peak eluting at about 50 min, as indicated by the arrow in Figure 1, showed any significant fluorescence. Subsequent amino acid analysis of this fluorescent peptide was as expected for the tryptic fragment 161–185, containing the Cys-177 residue [see Frank and Weeds (1974)].

Preparation of Labeled Subfragment 1. The incorporation of labeled A1 light chain into S1(A2) was readily accomplished by dissociation in 4.7 M NH_4Cl (Wagner & Weeds, 1977) but at the higher pH of 9.5, as suggested by Ueno and Morita (1984). The final hybridization mixture in 10-mL total volume consisted of 4.7 M NH_4Cl, 2 mM EDTA, 2 mM DTT, 0.9 M ammonia solution, 40 mg (0.37 μmol) of S1(A2), and 40 mg (1.93 μmol) of A1 light chain. All the components, with the exception of the S1(A2), were mixed, and the solution was stirred for about 2 min to let most of the light chain dissolve. The S1(A2) was then added and the reaction mixture stirred at 4 °C for 20 min. It was then immediately applied to Sephadex G-25 (1.6 × 55 cm) equilibrated in 10 mM MOPS and 0.25 mM DTT, pH 7.0. The resulting desalted protein fraction was then applied to a column of SP-Trisacryl (2 × 25 cm) equilibrated in the same buffer. The light chains, both labeled and unlabeled, passed through the column unretarded, and the bound S1 was subsequently eluted with a gradient of 0–0.2 M NaCl (2 × 100 mL) in 10 mM MOPS and 0.25 mM DTT, pH 7.0. Any remaining S1(A2) elutes first and is well separated from the S1(A1–AF) hybrid (Figure 2). The efficiency of exchange, as judged by the relative heights of the two peaks, varied between 30% and 60%. It was notable, however, that this increased to >80% when unlabeled light chain was used for hybridization. Similar results were obtained with 1,5-BrAEDANS- and 5-IAS-labeled light chains.

Cross-Linking of Subfragments 1 with pPDM. Reaction with pPDM to cross-link SH_1 and SH_2 thiols in the presence of 20 mM $MgCl_2$ and 0.15 mM ADP was carried out as

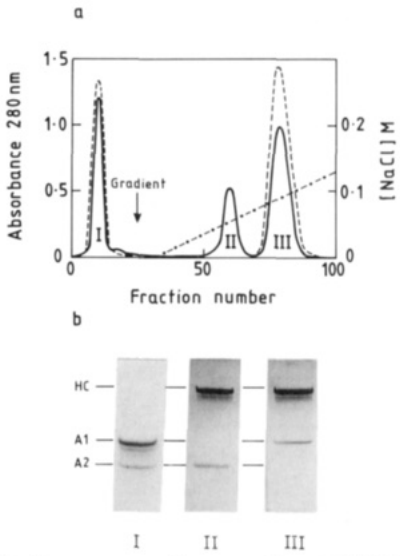

FIGURE 2: (a) Chromatographic separation of S1(A1–AF) formed from 40 mg of S1(A2) and 40 mg of A1–AF light chain. The protein was applied to a column (2.2 × 23 cm) of SP-Trisacryl equilibrated in 10 mM MOPS and 0.25 mM DTT, pH 7.0. Excess A1–AF light chain and A2 light chain passed through the column unretarded (peak I). The bound S1 was eluted with a gradient of 0–0.2 M NaCl (100 × 100 mL) in 10 mM MOPS and 0.25 mM DTT, pH 7.0. Protein was monitored at 280 nm (—) and the gradient determined by conductivity (–·–). Fluorescence emission was monitored at 520 nm following excitation at 495 nm (---). Column flow rate 40 mL·h^{-1}. Fraction volume 2.5 mL (b). Polyacrylamide gel electrophoresis in sodium dodecyl sulfate on 12% gels in Tris–Bicine buffer at pH 8.3 of peaks I, II, and III from (a). HC, S1 heavy chain; A1, alkali 1 light chain; A2, alkali 2 light chain.

described by Wells and Yount (1982). Excess reagents were removed by two successive precipitations with $(NH_4)_2SO_4$–EDTA mixtures followed by passage down Sephadex G-25, as described by these authors. The extent of nucleotide trapping was established by measuring the incorporation of Mg[^{14}C]ADP. This varied from 0.6 to 0.8 mol of ADP/mol of S1. Intramolecular cross-linking of SH_1 and SH_2 was also established by complete inactivation of the K^+-EDTA and Ca^{2+}-ATPase activities (Sekine & Kielley, 1964). In all cases, derivatization with pPDM was done after hybridization of the A1 light chain into S1.

Fluorescence Measurements. Steady-state fluorescence measurements were made by using a Spex Fluorolog spectrofluorometer equipped with a Datamate scan controller and data processor. The only exception to this was the fluorescence polarization measurements which were made by using a Baird Atomic SFR-100 spectrofluorometer fitted with two polaroid-coated filters with 10-nm slits in the paths of the excited and emitted beams (Chen & Bowman, 1965). In both cases, the emission spectra were corrected by using factors generated by a standard calibrated tungstem lamp. Energy transfer experiments were initially recorded with five different samples: (1) donor-labeled protein; (2) donor-labeled protein + unlabeled acceptor protein; (3) donor-labeled protein + acceptor-labeled protein; (4) acceptor-labeled protein + unlabeled donor protein; (5) acceptor-labeled protein. Samples 1–4 all contained identical concentrations of donor protein (labeled or unlabeled) while samples 2–5 contained identical concentrations of acceptor protein (labeled or unlabeled). Subsequently, however, it was found unnecessary to measure samples 4 and 5 since the fluorescence of the acceptor at the donor emission wavelength was found to be negligible. In order to minimize concentration quenching viz. inner filter effects and emission reabsorption, where possible, the optical densities at the excitation and emission wavelengths of samples used in fluorescence measurements were about 0.05 or less. For this reason, 0.5 cm^2 cuvettes were used in all experiments. This not only reduced the amount of sample required (0.6 mL) but also enabled the use of higher protein concentrations. The latter is particularly important for maximizing the amount of complex present in the weak binding states. Four further controls were included to validate the occurrence of true nonradiative energy transfer. First, samples were scanned in the presence of 6 M guanidine hydrochloride where no complex should be present and consequently no quenching of the donor fluorescence should be observed. Second, samples, where the optical density at the respective excitation and emission wavelengths exceeded 0.05, were diluted, and the donor quenching was remeasured. Third, donor quenching was measured at four different emission wavelengths, and finally, a wide range of protein concentrations was investigated using three donor–acceptor pairs. All fluorescence measurements were made at 25 °C in 10 mM TEA-HCl, 3.0 mM $MgCl_2$, 10 mM KCl, 1.5 mM EGTA, and 0.25 mM DTT, pH 8.0.

Determination of Energy Transfer. The efficiency of energy transfer, E, between a single donor and acceptor pair of chromophores is related to the distance between them, R, by

$$R = (1/E - 1)^{1/6}R_0 \quad (1)$$

R_0 is called the "Forster critical distance" and is the distance at which transfer efficiency is 50% (Forster, 1965). R_0 is related to the spectral properties of the chromophores by

$$R_0 = (8.79 \times 10^{-6})(Jk^2n^{-4}Q_D)^{1/6} \text{ nm} \quad (2)$$

where Q_D denotes the quantum yield of the donor fluorescence in the absence of transfer and n the index of refraction of the medium between the donor and acceptor. Since the latter is inaccessible in practice, a value of 1.4 is used (Beardsley & Cantor, 1970). k^2, the orientation factor, accounts for the relative orientation of the donor emission and acceptor absorption dipoles during transfer. The spectral overlap integral, J, is defined by

$$J = \int_0^\infty F_D(\lambda)E_A(\lambda)\lambda^4 \, d\lambda / \int_0^\infty F_D(\lambda) \, d\lambda \quad (3)$$

and calculated as the integrated mutual area of overlap between the donor emission spectrum (F_D) and the acceptor absorption spectrum (E_A). F_D is expressed in arbitrary fluorescence units, E_A in M^{-1} cm^{-1}, and wavelength (λ) in nanometers. The units for J are, therefore, nm^4 M^{-1} cm^{-1}. The transfer efficiency, E, was determined by measuring the quenching of donor fluorescence emission at a wavelength where there was no acceptor emission according to

$$E = 1 - Q_{DA}/Q_D \quad (4)$$

where Q_{DA} and Q_D are the relative donor quantum yields of the acto–S1 complexes in the presence and absence of acceptor, respectively. It was necessary to correct for nonstoichiometric protein labeling and for the presence of any uncomplexed donor-labeled protein as given by Takashi (1979). A detailed discussion of this method is given by Fairclough and Cantor (1978). The relative quantum yield of donor-labeled proteins, Q_D, was determined by reference to quinine sulfate as previously described (Trayer & Trayer, 1983). A value of $k_2 = {}^2/_3$ is used to calculate R_0 in eq 2 which assumes that the donor and acceptor chromophores are freely rotating and able to assume any orientation. In practice, however, this is very rarely the case, a problem which has been considered extensively by Dale et al. (1979). They have shown that limiting values can

Table I: Energy Transfer Parameters[a]

donor	acceptor	Q_D	$J \times 10^{15}$ (nm^4 M^{-1} cm^{-1})	$R_0(2/3)$ (nm)
S1(A1-AEDANS)	F-actin-AF	0.41	1.868	4.8
S1(A1-AEDANS)	F-actin-NBD	0.41	1.003	4.3
F-actin-AEDANS	S1(A1-AF)	0.49	1.647	4.8
F-actin-AS	S1(A1-AF)	0.22	1.647	3.5

[a] The procedures for calculating these parameters are given under Materials and Methods. Q_D is the quantum yield of the donor in the presence of unlabeled acceptor protein. J is the spectral overlap integral of the donor fluorescence and acceptor absorption calculated according to eq 3. $R_0(2/3)$ is the critical transfer distance at which the transfer efficiency is 50% and calculated according to eq 2.

be obtained for k^2 by measuring the emission anisotropy of the matrix-bound chromophores. The average depolarization factors, $\langle d \rangle$, for donor and acceptor chromophores bound to S1 and actin were measured as described elsewhere (Trayer & Trayer, 1983). The maximum and minimum values for k^2 were then calculated according to Dale et al. (1979), and from these, a range of values for R_0 can be estimated by using the equation:

$$R_{0,\text{max or min}} = (1.5\langle k^2 \rangle_{\text{max or min}})^{1/6} R_0(2/3) \qquad (5)$$

By substitution of these values in eq 1, a range of distances, R, between the two sites can be determined.

Results

ATPase Activities. Hybridized S1 containing the labeled A1 light chain showed no impairment of any ATPase activities when compared to native S1(A1). According to the conditions detailed under Materials and Methods, the Ca^{2+}-ATPase activity was 3.8 (±0.6) s^{-1}, and the K^+-EDTA ATPase activity was 12.5 (±1.2) s^{-1}. Examination of the Mg^{2+}-ATPase activity, obtained as a function of increasing F-actin concentration, gave a V_m of 25 (±3.1) s^{-1} and a K_{app} of 28 (±4.2) μM. When F-actin labeled at Cys-374 was used, the V_m remained relatively unchanged, but the K_{app} was generally slightly reduced, giving a value of 19 (±5.1) μM. The values in parentheses are standard error of mean values obtained over many different preparations and labeling of S1(A1) and actin. Modification of S1 with pPDM completely abolished all ATPase activity.

Energy Transfer Parameters. The quantum yields (Q_D) of donor-labeled proteins in the presence of acceptor protein, together with the corresponding overlap integrals (J), are given in Table I. Estimates of $R_0(2/3)$ were calculated according to eq 2 assuming a refractive index of 1.4 and a value for k^2 of $^2/_3$.

Limiting Values for k^2 and R_0. Calculation of R_0 using a value of $^2/_3$ for k^2 assumes isotropic distribution for the transition moments of the donor and acceptor chromophores. If this were the case, then the average depolarization factor, $\langle d \rangle$, would be zero. It is clear from the results shown in Table II that this is not the case. There is, however, some reorientation following excitation since $\langle d \rangle \neq 1$. From the donor and acceptor polarization factors, limiting values for k^2 were calculated. These are given in Table II, together with the corresponding maximum and minimum values for R_0. These values represent the extreme range of R_0 values for the particular donor–acceptor pair.

Energy Transfer in the Strong Binding Acto–S1 Complex. Measurements were made between regulated actin labeled at Cys-374 on actin and S1(A1) labeled at Cys-177 on the A1 light chain, using three different donor–acceptor pairs of fluorescent chromophores. The molar ratio of actin to Tn–TM was maintained at 7:2. Samples of actin or S1 labeled with 1,5-BrAEDANS as the donor were excited at 360 nm. Energy transfer was estimated by measuring the donor emission at 10-nm intervals from 450 to 480 nm as described under Materials and Methods. With 5-IAS as the donor chromophore, excitation was at 310 nm, and donor emission was measured from 425 to 455 nm every 10 nm. Direct binding measurements on samples used in energy transfer showed that in all cases >95% of S1 was bound to actin.

The results obtained with several different protein preparations and labelings are summarized in Table III, and each value is the average of at least five determinations. It can be seen that under these conditions, where the affinity of S1 for actin is very high, the spatial separation of the two chromophores is about 6 nm, agreeing well with our earlier published observations (Bhandari et al., 1985). The transfer efficiency between F-actin–AS and S1(A1–AF) of around 4% is really too low for an accurate estimation of R. This low transfer efficiency is only to be expected, however, with an R_0 of 3.5 nm when the two probes are some 6 nm apart. No differences in transfer efficiency could be detected in the presence (0.5

Table II: Limiting Values of k^2 and R_0[a]

donor	acceptor	$\langle d \rangle$ donor	$\langle d \rangle$ acceptor	k^2 min	k^2 max	R_0 (nm) min	R_0 (nm) max
S1(A1-AEDANS)	F-actin-AF	0.403	0.491	0.221	2.448	3.99	5.96
S1(A1-AEDANS)	F-actin-NBD	0.403	0.527	0.213	1.829	3.56	5.09
F-actin-AEDANS	S1(A1-AF)	0.638	0.465	0.173	2.077	3.83	5.79
F-actin-AS	S1(A1-AF)	0.501	0.465	0.203	1.892	2.87	4.16

[a] The average depolarization factors ($\langle d \rangle$) and orientation factor (k^2) were determined as described under Materials and Methods and calculated according to Dale et al. (1979). The max and min values for R_0 were calculated according to eq 5.

Table III: Energy Transfer in a Strong Binding Complex of Regulated Acto-S1[a]

donor	acceptor	$R_0(2/3)$ (nm)	E (%)	$R(2/3)$ (nm)	R (nm) min	R (nm) max
S1(A1-AEDANS)	F-actin-AF	4.8	17 (±1.5)	6.3	5.2	7.8
S1(A1-AEDANS)	F-actin-NBD	4.3	14 (±1.7)	5.8	4.8	6.9
F-actin-AEDANS	S1(A1-AF)	4.8	28 (±2.1)	5.6	4.5	6.8
F-actin-AS	S1(A1-AF)	3.5	4 (±2.5)	6.0	4.7	6.8

[a] The transfer efficiencies (E) were measured as described under Materials and Methods and calculated according to eq 4 and Takashi (1979). Each value for E (±SE) was the average of at least five determinations. The protein concentrations were varied between 1 and 20 μM, and the actin:S1 ratio varied between 1 and 11. Actin was mixed with Tn–TM complex in a molar ratio of 7:2, respectively. All measurements were made at 25 °C in 10 mM TEA-HCl, 10 mM KCl, 3 mM $MgCl_2$, and 0.25 mM DTT, pH 8.0.

Table IV: Energy Transfer in a Complex of Regulated Actin and pPDM·S1[a]

donor location	donor concn (μM)	acceptor pPDM·S1(A1–AF) concn (μM)	complex concn (μM)	E (%)	$R(2/3)$ (nm)	R (nm) min	R (nm) max
F-actin–AEDANS	8	2	0.46	>95	≤2.8		
	10	1	0.24	>95	≤2.8		
	10	5	0.78	>95	≤2.8		
F-actin–AS	6	3	0.39	80	2.8	2.3	3.3
	10	1	0.21	85	2.6	2.2	3.1
	20	5	1.60	93	2.3	1.9	2.7
	20	7	2.20	83	2.7	2.4	3.5

[a] Regulated actin comprised actin and Tn–TM complex mixed in a molar ratio of 7:2, respectively. The concentration of the transferring species (complex concn) was determined by spinning down as described under Materials and Methods. Conditions used: 10 mM TEA-HCl, 10 mM KCl, 3 mM $MgCl_2$, and 0.25 mM DTT, pH 8.0 at 25 °C.

mM $CaCl_2$) or absence (1.5 mM EGTA) of calcium.

By substitution of the maximum and minimum values for k^2 into eq 5, the corresponding maximum and minimum values for R_0 were calculated. These values for $R_{0,min}$ and $R_{0,max}$ were then used in eq 1 to calculate a range of distances, R, between the two sites. Although on first inspection these ranges may seem quite large, it must be remembered that these values represent the absolute bounds of R values, and in general, it is unlikely that the actual distance lies at the extremes of this range. Given that $R_{max}/R_{min} = k^2_{max}/k^2_{min}$ (Eisinger, 1976), the corresponding uncertainty in R was estimated to be about 18–20%.

Energy Transfer between Regulated Actin and pPDM·S1. Measurements were made using a single acceptor chromophore, 5-IAF, covalently attached to pPDM·S1 via Cys-177 on the A1 light chain. Either one of two donor chromophores, namely, 1,5-BrAEDANS or 5-IAS, was attached to actin at Cys-374. Samples in which BrAEDANS was the donor were excited at 360 nm, and the donor emission was measured at 450, 460, 470, and 480 nm, while IAS-labeled actin was excited at 310 nm and the donor emission evaluated at 425, 435, 445, and 455 nm. The results of several experiments are shown in Table IV.

With AEDANS as the donor, the transfer efficiency was always close to 100%, confirming our original observations (Bhandari et al., 1985). From this value, R can be estimated to be less than 3 nm. However, since E is only a sensitive function of R over the range $0.5R_0 < R < 1.9R_0$ [see Fairclough and Cantor (1978)], a distance of about 3 nm is at the extreme end of this range where the functional dependence of E on the distance R is not very accurate. To enable more precise limits to be placed on the magnitude of the change observed, F-actin labeled with 5-IAS was used as the donor molecule and pPDM·S1(A1–AF) as the acceptor. The corresponding value for $R_0(2/3)$ was calculated to be 3.5 nm (Table I).

An example of energy transfer between F-actin–AS and pPDM·S1(A1–AF) is illustrated in Figure 3. The presence of energy transfer can be detected by comparing the labeled acto–S1 complex (scan 3) with the donor-labeled complex (scan 2). A decrease in donor emission is clearly observed. In the absence of the acceptor probe, however, addition of unlabeled pPDM·S1(A1) to F-actin–AS causes an increase in donor emission (scan 2) compared to F-actin–AS alone (scan 1). It is important to note that in scan 2 the unlabeled S1 had been cross-linked with pPDM. This applies to all the experiments summarized in Table IV. Separate experiments comparing S1(A1) cross-linked with pPDM to derivatives of S1(A1) that had been hybridized with unlabeled A1 light chains prior to cross-linking with pPDM did not reveal any significant difference either in the affinity of the S1(A1) derivative for F-actin or in changes in the fluorescence emission

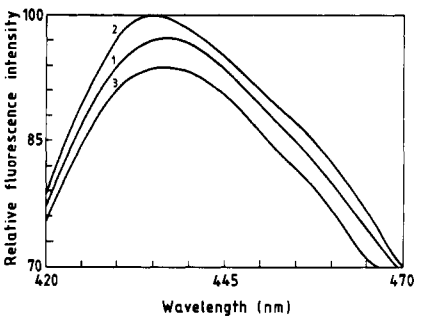

FIGURE 3: Corrected fluorescence emission spectra of regulated F-actin–AS (0.8 mol of label/mol of protein) and pPDM·S1(A1–AF) (0.6 mol of AF/mol of protein) in the absence of calcium. Samples were excited at 310 nm, and all measurements were made in 10 mM TEA-HCl, 3 mM $MgCl_2$, 10 mM KCl, 1.5 mM EGTA, and 0.25 mM DTT, pH 8.0 at 25 °C. Scan 1, regulated F-actin–AS, 20 μM; scan 2, regulated F-actin–AS, 20 μM, + pPDM·S1(A1), 7 μM; scan 3, F-actin–AS, 20 μM, + pPDM·S1(A1–AF), 7 μM. In all samples, Tn–TM complex concentration was 5.7 μM. The concentration of regulated acto–S1 complex present in scans 2 and 3 was determined by direct binding measurements to be 2.2 μM.

when added to labeled actin. From Table IV, it can be seen that even with this pair of chromophores the energy transfer efficiency is still very high, giving values for R of around 2.5 nm. Addition of ATP to a final concentration of 1.5 mM did not produce any change in the values of E obtained, emphasizing that the pPDM cross-linking was complete and that no rigor complex was present.

In these and subsequent experiments, only small fluorescence changes were expected because of the smaller amount of actin-bound S1 in the weakly bound complex. Hence, the experiments were repeated over a range of protein concentrations and on at least three pairs of protein preparations, giving rise to donor quenching in the range of 1.5–10% over the whole data set. Illustrated in Figure 3 is donor quenching of F-actin–AS of some 6%, which can be seen to be readily measurable. Reproducible results were consistently obtained. Donor quenching was eliminated when the measurements were repeated in 6 M guanidine hydrochloride.

The experiment quoted in Table IV, containing 10 μM F-actin–AEDANS and 5 μM pPDM·S1(A1–AF), had an absorption value at the donor excitation and emission wavelengths >0.05 but <0.1. Dilution with an equal volume of buffer to 5 μM F-actin–AEDANS and 2.5 μM pPDM·S1-(A1–AF) gave a complex concentration of 0.26 μM and reduced the respective optical densities to <0.05 with no concomitant change in energy transfer, i.e., $E > 95\%$. Samples where F-actin–AS was used as the donor provided no problem with high optical densities because donor emission was measured at wavelengths further away from fluorescein absorption. This was confirmed by diluting the samples containing 20 μM F-actin–AS 3-fold and remeasuring the complex concentration

Table V: Energy Transfer in a Complex of Regulated Acto-S1 in the Presence of ATP and the Absence of Calcium[a]

donor location	concn (μM)	acceptor location	concn (μM)	complex concn (μM)	E (%)	$R(2/3)$ (nm)	R (nm) min	R (nm) max
S1(A1-AEDANS)	2.3	F-actin-NBD	10.7	0.27	80	3.4	2.8	4.0
	4.1		4.4	0.45	89	3.0	2.5	3.6
S1(A1-AEDANS)	3.0	F-actin-AF	6.4	0.20	90	3.1	2.8	4.1
	3.6		4.2	0.22	86	3.5	2.9	4.5
F-actin-AS	10.0	S1(A1-AF)	1.0	0.19	71	3.0	2.5	3.6
F-actin-AEDANS	11.2	S1(A1-AF)	1.0	0.25	>95	≤2.8		
	10.0	S1(A1-AF)	1.0	0.21	>95	≤2.8		
	6.5		3.2	0.49	>95	≤2.8		
	3.2		4.2	0.31	>95	≤2.8		

[a] All measurements were made at 25 °C in 10 mM TEA-HCl, pH 8.0, containing 10 mM KCl, 3 mM $MgCl_2$, 0.25 mM DTT, 1.5 mM EGTA, and 1 mM ATP. The concentration of the acto-S1 complex present during energy transfer was determined by spinning down as described under Materials and Methods. Actin was mixed with Tn-TM complex in a molar ratio of 7:2, respectively.

and donor quenching. Transfer efficiency, E, in these diluted samples was the same as that quoted for the more concentrated samples in Table IV.

Crucial to all measurements of E is a knowledge of the amount of acto-S1 complex and hence transferring species present. For this reason, binding studies were carried out directly on the same samples used for the transfer experiments and on a parallel set of samples at concentrations identical with those used in energy transfer. The amount of complex present for each experiment is given in Table IV. It was generally observed that between 15% and 30% of the available S1 was complexed to actin compared to >95% of un-cross-linked S1(A1). Estimation of binding constants of pPDM·S1 for actin from these data gave values in the range of (2–4) × 10^4 M^{-1}. Similar values were obtained for the binding of S1·ATP to regulated actin by Chalovich and Eisenberg (1982). The results presented here clearly indicate that when actin and S1 form a weakly associated complex, fluorescent probes, located on the A1 light chain in S1 and at the penultimate residue in actin, move close together by some 3 nm when compared to an equivalently modified strong binding complex.

Energy Transfer between Regulated Actin and S1 in the Presence of ATP and the Absence of Calcium. An example of energy transfer between S1(A1-AEDANS) (donor) and regulated F-actin-NBD (acceptor) in the absence of calcium (1.5 mM EGTA) and the absence and presence of ATP is illustrated in panels a and b, respectively, of Figure 4. Following excitation of the donor at 360 nm, the fluorescence emission was scanned over the range 450–550 nm. The presence of energy transfer can be detected by comparing the fluorescence intensity at a wavelength (450–480 nm) containing only donor and no acceptor emission; i.e., the donor emission intensity is less in the doubly labeled acto-S1 complex (scan 3) than in the singly labeled acto-S1 complex (scan 2). It can also be seen that while the addition of acceptor-labeled regulated F-actin to S1 (A1-AEDANS) results in a decrease in donor emission as a direct result of energy transfer, addition of unlabeled regulated F-actin (scan 2) increases the donor emission when compared to S1(A1-AEDANS) alone (scan 1). In the absence of ATP, the measured transfer efficiency of 14% gave a distance of 5.8 nm between the two labeled sites (Figure 4a and Table III). Under these conditions, all of the S1 was complexed to actin. Following addition of 1 mM ATP, the samples were immediately rescanned such that donor emission was recorded within 2 min (Figure 4b). The amount of acto-S1 complex present was estimated by spinning down in the Airfuge immediately after scanning. A parallel set of samples, identical with those used to determine energy transfer, were also spun down immediately after addition of 1 mM ATP, without scanning. No significant difference was observed in

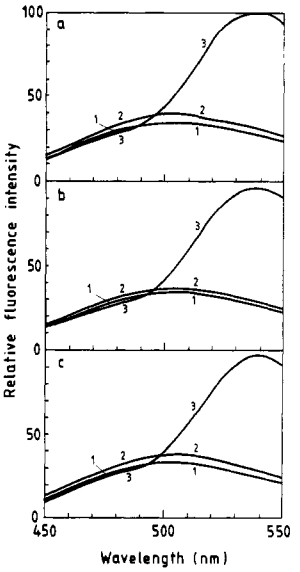

FIGURE 4: Corrected fluorescence emission spectra of regulated F-actin-NBD and S1(A1-AEDANS) in the absence of calcium and the presence and absence of ATP. Samples were excited at 360 nm, and all measurements were made in 10 mM TEA-HCl, 3 mM $MgCl_2$, 10 mM KCl, 1.5 mM EGTA, and 0.25 mM DTT, pH 8.0 at 25 °C. Scan 1, S1(A1-AEDANS), 2.3 μM; scan 2, S1(A1-AEDANS), 2.3 μM, and regulated F-actin, 10.7 μM; scan 3, S1-(A1-AEDANS), 2.3 μM, and regulated F-actin-NBD, 10.7 μM. In all samples, Tn-TM complex concentration was 3 μM. (a) Emission spectra in the absence of ATP. (b) Samples from (a) rescanned immediately after the addition of 1 mM ATP. (c) Samples from (b) rescanned after 45 min at 25 °C. Concentration of acto-S1 in (a), (b), and (c) was 2.4, 0.27, and 1.9 μM, respectively. Actin and S1 were labeled at 1 mol of fluorophore/mol of protein.

the amount of complex present, namely, 0.27 μM. A transfer efficiency of 80% was calculated by using this value, giving a spatial separation between the donor and acceptor chromophores of 3.4 nm (Table V). This represents a decrease in distance of 2.4 nm, compared to the distance of 5.8 nm found for the strong binding complex (Figure 4a, Table III).

The hydrolysis rate of MgATP by S1(A1-AEDANS) at 25 °C with a 5–10-fold molar excess of regulated F-actin or F-actin-NBD in the absence of calcium was about 0.18 s^{-1} compared to 1.7 s^{-1} in the presence of calcium (0.5 mM $CaCl_2$). (Values given are not corrected for hydrolysis by S1 alone of 0.1 s^{-1}.) Consequently, only about 5% of the available MgATP was estimated to be hydrolyzed before the samples shown in Figure 4b were scanned. The samples containing ATP were then left at 25 °C for 45 min, when all of the ATP was judged to be hydrolyzed and rescanned (Figure 4c). After this time, the amount of complex present was found to be 1.9 μM. Calculation of transfer efficiency showed a decrease to

12%, indicating that the two probes had reverted to their original "rigor" distance of about 5.9 nm. These findings were further substantiated by using all three pairs of donor–acceptor chromophore-labeled proteins over the range of protein concentrations given in Table V. It should be noted that in all cases donor quenching was eliminated in the presence of 6 M guanidine hydrochloride.

Table V summarizes the data obtained for energy transfer in the weak binding complex of regulated acto–S1 in the presence of ATP and the absence of calcium. Good agreement was found between the three pairs of fluorophores used over a range of donor:acceptor ratios. In samples containing concentrations of the protein–fluorescein acceptor >4 μM, the absorption at the donor emission wavelength was >0.05 but <0.1. In one case, i.e., 3 μM S1(A1–AEDANS) and 6.4 μM F-actin–AF, the total absorption at 480 nm was 0.13. Under these circumstances, it is feasible that donor quenching may result from direct radiative absorption of donor emission by the acceptor and not by true nonradiative energy transfer. We are confident, however, that this is not the case for the following reasons. (i) Samples at the same protein–probe concentrations in 6 M guanidine hydrochloride exhibited no energy transfer. (ii) Estimation of E at donor emission wavelengths where the optical density was <0.05 gave the same results. (iii) The whole data set is internally consistent and in agreement with that in Table IV. We did not attempt direct dilution of these samples as the diminishing amounts of complex present would make measurements difficult. When, however, equivalent concentrations of the strong binding complex (i.e., prior to the addition of ATP) were diluted out as much as 5-fold, no significant changes in E were ever obtained.

These observations confirm that energy transfer is occurring and clearly demonstrate that in the weak binding complex formed in the presence of ATP and the absence of calcium between regulated actin labeled at Cys-374 and S1(A1) labeled at Cys-177 on the A1 light chain, there is a large increase in transfer efficiency and hence a decrease in distance between the two sites, when compared to the strong binding complex (Table III). Estimation of the approximate association constants from the binding data for the amounts of complex present in Table V ranges from 1×10^4 to 3×10^4 M^{-1}, which agrees well with the values reported by Chalovich and Eisenberg, (1982). It should be noted that the distances between the two sites [$R(2/3)$] shown in Table V are similar to those obtained for the acto–pPDM·S1 complex (Table IV) and confirm that the latter is a good analogue of the S1·ATP state.

Discussion

The results presented in this report confirm and considerably extend the observations made in an earlier paper that there is a significant structural difference between the weak binding acto–S1–ATP complex and the rigorlike acto–S1 complex (Bhandari et al., 1985). In both of these studies, FRET measurements have been made between fluorescent probes located at Cys-374 of actin and Cys-177 of the A1 light chain on S1(A1). In the rigor complex, three different pairs of donor–acceptor chromophores have now been employed (Table III) and establish that the distance [$R(2/3)$] between the two sites is some 6 nm. Reversing the donor–acceptor pair in one case does not alter this result. Rather interestingly, with one donor–acceptor pair, i.e., 5-IAS and 5-IAF, which has an $R_0(2/3)$ = 3.5 nm, FRET is barely detectable in this strong binding complex, much as would be anticipated from the other results.

In this report, two strategies have been employed to "arrest" the contractile cycle in the weak binding, pre-power stroke state. Our initial experiments were performed with actin and pPDM-cross-linked S1 as in our earlier studies (Bhandari et al., 1985). This S1 derivative has been shown to bind to actin with the same affinity and binding characteristics as S1·ATP (Greene et al., 1986). Initial observations with pPDM·S1–actin were made with 1,5-BrAEDANS as the donor and 5-IAF as the acceptor [$R_0(2/3)$ = 4.8 nm]. In these experiments, the donor fluorescence was almost entirely quenched, giving a value for transfer efficiency of $E > 95\%$, indicating a spatial separation of $\leq$2.8 nm between the two probes, and showing that the two sites had moved closer by some 3 nm (Table IV; Bhandari et al., 1985). This distance is at the extreme end of the range for this particular donor–acceptor pair; hence, E will not be particularly sensitive to changes in R. Ideally, R_0 should be equal to or close to the estimated distance. For this reason, the experiments were repeated using a second pair of chromophores with a lower $R_0(2/3)$, namely, 5-IAS and 5-IAF. With pPDM·S1–actin, the value of E was between 89% and 90%, giving a value for R of between 2.3 and 2.8 nm. Although these results substantiate our earlier observations (Bhandari et al., 1985), the transfer efficiency is still very high, which suggests that the two sites may be even closer. Addition of ATP to complexes of actin and pPDM·S1 had no effect on either the value of E or the amount of complex formed in parallel binding studies. This observation demonstrates that there is no un-cross-linked S1 in the pPDM·S1 preparation, giving rise to rigor acto–S1 complexes which would interfere with the results.

Whereas pPDM·S1 does appear to be a very good analogue of S1·ATP, there is always the concern that cross-linking the two essential thiols may induce conformational constraints not immediately apparent. For this reason, the experiments were repeated adopting a second method, whereby a stable weak binding acto–S1 complex was formed without resort to further chemical modification. It has been observed by Greene et al. (1986) that S1·ATP forms a weak binding complex with regulated actin and if calcium ions are absent then the rate of ATP hydrolysis is extremely low. Hence, a relatively long-lived weak binding acto–S1 complex is generated which closely represents the situation in relaxed muscle. With the use of this complex, FRET was monitored with three pairs of donor–acceptor chromopores with a range of R_0 values. The results obtained (Table V) supported our observations using pPDM·S1–actin, such that in this weak binding complex the probes are again some 3 nm apart. The values for $R(2/3)$ in Table V are slightly higher than those for pPDM·S1–actin in Table IV, although when uncertainties in k^2 are taken into account, the R_{min} and R_{max} values overlap considerably, suggesting any differences are within experimental error. It is feasible, however, that the configuration of the more "native" weak binding state may be slightly different from that generated by pPDM-cross-linking of S1. Nevertheless, the pPDM·S1 derivative would appear to be a good analogue of the S1·ATP state from these data.

When calculating intramolecular distances using FRET measurements, one of the most crucial factors is the amount of complex present during transfer. In a strong binding complex where the affinity of S1 and actin is very high ($K_a > 10^6$ M^{-1}), this is not difficult to determine. In the situation of a weak binding complex, however, where the affinity is considerably reduced ($K_a \sim 10^4$ M^{-1}), precise estimation of the amount of complex present becomes of paramount importance, particularly at the relatively low protein concentrations used in FRET experiments. Consequently, the amount of acto–S1 complex present was determined for each set of

energy transfer data obtained. Binding studies were carried out on the same set of samples used to estimate energy transfer and on a parallel set of identical samples at the same time as the fluorescence measurements were made. It was felt that the latter may be particularly important for samples following addition of ATP, although no significant differences between the two data sets were found.

Considerable support for these observations comes from ^1H NMR studies comparing the binding of S1 and pPDM·S1 to actin. A feature of the ^1H NMR spectrum of S1 as it binds to actin is the large apparent loss of signal intensity under the CH_3–CH_2 envelope, presumed to signify a loss of mobility of the amino acid side chain residues on complex formation (Prince et al., 1981; Highsmith et al., 1979). In contrast, pPDM·S1 binds to actin without this extensive loss of side chain mobility (Goodearl et al., 1985). At the higher protein concentrations used in these ^1H NMR experiments, 85% of the pPDM–S1 was bound to the actin. Thus, different conformations of actin-bound S1 exist in the two states, consistent with the significant change in distance between Cys-374 of actin and Cys-177 of the A1 light chain in S1(A1) noted above.

One of the most difficult parameters to quantify in FRET studies is the orientation factor k^2. In the absence of any definitive information, it is generally assumed that both donor and acceptor fluorophores are in a state of rapid and unconstrained motion and as such $k^2 = {}^2/_3$. This approximation becomes questionable when the chromophores are covalently bound to proteins, and from our earlier studies (Trayer & Trayer, 1983), it has been shown that this is certainly not the case in this system. Nevertheless, an estimate of possible limiting values for k^2 and hence R_0 and R can be obtained from measurements of the limiting depolarization factors, $\langle d \rangle$ (Dale et al., 1979). In agreement with our earlier findings, values obtained for $\langle d \rangle$ suggested that while the fluorophores were not randomly oriented neither were they immobile. From these values, R_{mean}, the average between R_{min} and R_{max}, can be estimated with an uncertainty of about 20% (Eisinger, 1976). Comparison of the results in Tables IV and V with those in Table III, however, shows that no overlap occurs between the extreme ranges of R_{min} and R_{max} in the two states. Thus, the results presented here demonstrate that a considerable shift in distance (with an estimated mean of 3 nm) between a point on S1 and point on actin occurs when changing from a weak to a strong binding state.

Previous studies on the structure of the acto–S1 complex using FRET have concentrated on the strong binding complex with actin labeled at Cys-374 and S1 modified on the heavy-chain SH_1 site (Takashi, 1979; Trayer & Trayer, 1983). Recently, Arata (1986) has attempted to study FRET between these two sites but in the presence of ATP, i.e., the weak binding complex. In order to stabilize the weak binding state, the two proteins were covalently cross-linked with a carbodiimide in the presence of ATP. An increase of about 0.6 nm was observed when compared to the "rigor" complex. It was, however, noted that if actin and S1 were cross-linked in the absence of ATP and the latter added subsequently, no significant change in transfer efficiency was detected. This is somewhat disconcerting in light of recent electron microscopy studies (Craig et al., 1985) which seem to show that addition of MgATP to carbodiimide-cross-linked acto–S1 evokes a definite change in structure of the attached S1.

A possible explanation of these observations is our recent report that a 30-residue peptide containing both the SH_1 and SH_2 fast-reacting thiols binds to actin just C-terminal to the SH_1 thiols (Cys-705) (Griffiths et al., 1987). Thus, this Cys residue is placed close to a major site of interaction between actin and the myosin head, and any reorientation of S1 around this point would not be expected to lead to any gross change relative to any point on actin. This is especially true since it has been observed that S1 and pPDM·S1 bind equally well (as judged by ^1H NMR) to a partial cyanogen bromide fragment from the N-terminus of actin (residues 1–82) (Moir et al., 1987). Since the 30-residue S1 heavy-chain peptide also binds to the N-terminal region of actin (B. A. Levine, A. G. Moir, and I. P. Trayer, unpublished observations), these data suggest that the region C-terminal to Cys-705 interacts with the actin in both the weak binding and strong binding complexes. A relatively immobile "domain" around the SH_1 thiol in isometrically contracting muscle has been invoked by Thomas and Cooke (Cooke et al. 1982, 1984) to explain their EPR measurements of myosin head orientation using muscle fibers in which the SH_1 was modified with a nitroxyl spin-label. These workers found only one class of immobilized myosin heads with an orientation similar to that found in rigor.

A number of recent observations by probe and X-ray methods on the behavior of cross bridges during contraction have been interpreted in terms of cycling between strongly and weakly attached actomyosin states [see, for example, Huxley and Kress (1985), Cooke (1986), and Eisenberg and Hill (1985) and references cited therein]. In these models, the strongly bound state at the end of the power stroke is considered to represent the angled attachment of the myosin head similar to that seen in rigor and acto–S1 in solution. The pre-power stroke weakly attached state is currently considered to be a loosely attached state, perhaps involving a flexible and internally mobile form of the myosin head, where the head can adopt several angles of attachment to actin. This may be true for contracting muscle where the geometric and mechanical constraints of the filament lattice will operate. It is less likely to be true in solutions, as the weakly bound S1·ATP might be expected to bind in an orientation representing the lowest free energy state. Given this assumption, then the results presented here could be interpreted as consistent with the rocking cross-bridge model.

It remains to be decided how much of the distance changes noted here can be represented by relative movements between the myosin head and actin and how much is occurring intrinsically within the myosin head. It has already been noted that the alkali light chain readjusts its structure as a consequence of actin binding to the 20-kDa domain of S1 in the isolated 20-kDa A2 fragment (Chaussepied et al., 1986). Furthermore, structural changes in thick filaments as a result of ATP binding (in the absence of actin) have also been noted (Clarke et al., 1986). If it is considered that S1 has a domain structure (Mornet et al., 1981) with regions of high segmental mobility (Prince et al., 1981), then both types of movement are likely to occur. As a result of ATP binding/hydrolysis, some rejuxtapositioning of the myosin head domains could result in a reorientation of S1 relative to the actin filament. These result do suggest, however, that there is enough structural information in the myosin head to account for the tension-generating process without having to invoke any other part of the molecule. This is consistent with the recent results using model systems where actin filaments have been shown to move in the presence of ATP on immobilized S1 (Toyoshima et al., 1987).

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