Resonance energy transfer evidence for two attached states of the actomyosin complex

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Received 21 May 1985

Resonance energy transfer measurements were made between a donor fluorophore, *N*-(bromacetyl)-*N'*-(1-sulpho-5-naphthyl)ethylenediamine, located on the single cysteine of the Al light chain of myosin S1(A1), and an acceptor fluorophore, 5-(iodoacetamido)fluorescein, sited on Cys-374 of actin. In the binary rigor complex a transfer efficiency of 24% was noted, representing a spatial separation of about 6 nm. When the same measurements were made using a stable analogue of S1·ATP, in which the fast reacting SH_1 thiol group is crosslinked to another thiol group in the 20 kDa domain of S1, the 2 fluorophores were found to have moved closer together by $\geqslant 3$ nm. This provides, for the first time, direct experimental evidence for a change in structure of the myosin crossbridge that could account for tension generation.

Actin-myosin interaction *Muscle contraction* *Crossbridge cycling* *Resonance energy transfer* *Subfragment 1*

1. INTRODUCTION

The model [1] that has provided the most convenient framework within which to consider mechanical and kinetic aspects of myosin crossbridge behaviour [2,3] during muscle contraction suggests that force generation involves a change in orientation of the myosin heads relative to the thin filament. The success of this model has tended to obscure the fact that direct experimental evidence about the actual structural changes that crossbridges undergo during tension development has been very difficult to obtain. Time-resolved X-ray diffraction patterns have come the closest to providing such evidence [4], but have been unable to clarify the detailed nature of the attached states. On the other hand, Cooke et al. [5] have monitored the orientation of spin labels specifically attached to the reactive sulphydryl (SH_1) on myosin heads in glycerinated muscle and found evidence for only one attached state in isometric tension, the same as that found in rigor muscle. To identify a second attached state, distinct from the rigor state, it is necessary to find some means of arresting the contractile process at the pre-power stroke stage. From an analysis of the binding of S1-nucleotides to actin, Eisenberg and Greene [6] have concluded that the strongly bound states (acto-S1, acto-S1–ADP) are associated with the end of the power stroke ('rigor' condition) and that the weakly bound states (acto-S1–ATP or acto-S1–ADP·P_i) are associated with the beginning of the power stroke. A stable analogue of S1·ATP has been described in which the SH_1 thiol

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Abbreviations: S1, myosin subfragment 1; S1(A1), S1(A2), rabbit fast-twitch muscle myosin subfragment 1 containing either the alkali 1 (A1 or LC1) light chain or the alkali 2 (A2 or LC3) light chain; BrAEDANS, *N*-(bromoacetyl)-*N'*-(1-sulpho-5-naphthyl)ethylenediamine; IAF, 5-(iodoacetamido)fluorescein; *p*PDM, *p*-phenylenedimaleimide; Ado*PP*[NH]*P*, adenosine 5'-[β,γ-imido]triphosphate

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00145793/85/$3.30

is crosslinked to another thiol group in the 20 kDa domain of S1, entrapping the nucleotide at the active site [7]. This derivative possesses actin-binding properties very similar to S1 · ATP [8] and so provides a means of arresting the contractile process at the pre-power stroke stage.

Here we report resonance energy transfer experiments between selected points on actin and on the isolated myosin head (subfragment 1, S1) that show a change of $\geqslant 3$ nm in distance when measurements are compared between the binary (rigor) acto-S1 complex and the thiol-crosslinked analogue of the ternary actin-S1–ATP complex. This provides, for the first time, direct experimental evidence for a change in structure of the myosin crossbridge that could account for tension generation.

2. MATERIALS AND METHODS

2.1. *Protein preparations and labelling protocols*

Myosin, S1(A1), S1(A2) and actin were prepared as described in [9] and light chains were extracted from myosin by the urea method and fractionated as cited in [10]. Protein concentrations were estimated by absorbance measurements using the coefficients given in [10]. The single cysteine (Cys-177) of the A1 light chain was reacted in the isolated light chain with BrAEDANS [11] and the extent of labelling determined by absorption and fluorescence emission [11]. This conjugate was hybridized into S1(A2) largely by the procedures outlined by Sivaramakrishnan and Burke [12] except that the reaction products were separated on a Matrex gel blue A column (Amicon, Lexington, MA). The proteins were applied to a 12 × 1.3 cm column in 50 mM imidazole-HCl (pH 7.0), 0.25 mM dithiothreitol and eluted with a gradient to 0.2 M NaCl in this buffer (total volume, 300 ml). The hybrid S1(A1-AEDANS) was the last species to elute. After concentration, this was passed down a Sephadex G-25 column in 0.5 M KCl, 50 mM Tris-HCl (pH 9.0) and 0.5 mM dithiothreitol to ensure that no Cibacron blue dye was present in the S1. This hybrid was used in the experiments described in sections 3.1 and 3.2. Samples were further derivatized by reaction with *p*PDM to crosslink the SH_1 and SH_2 thiols in the presence of 20 mM $MgCl_2$ and 0.1 mM ATP exactly as described by Wells and Yount [13]. This material was used in the experiments described in section 3.3.

Actin was labelled as G-actin with IAF and, after removal of excess chromophore it was polymerised to F-actin [9]. The specificity of fluorophore labelling of all of these proteins was confirmed by peptide studies [14] and their enzymic activities (where appropriate) were shown to be unimpaired by these procedures.

2.2. *Fluorescence methods*

All measurements were made at 25°C in cells of 5 mm path length (sample volume 0.6 ml) using a Spex fluorolog spectrofluorimeter, with a Datamate spectrometer controller and data processor (Spex Industries). The total absorbance at the exciting wavelength was always <0.07. The absence of a trivial absorption was demonstrated by the fact that the emission intensity increased linearly from the origin over the range of protein concentrations used here whereas the measured transfer efficiencies remained constant. Resonance energy transfer efficiencies were estimated by donor quenching [15] and details of data calculations are given in [9,16]. Experimental protocols and the spectral parameters used in determining the transfer efficiency are given in the figure and table legends.

2.3. *Analytical procedures*

The amount of complex formed between F-actin-AF and *p*PDM-S1(A1-AEDANS) was determined by spinning down the complex in a Beckman Airfuge [17]. Samples from the fluorescence measurements (180 μl) were centrifuged at 178000 × *g* for 40 min and the concentration of S1(A1-AEDANS) in the supernatant determined by fluorescence. Protein purity was routinely monitored by polyacrylamide gel electrophoresis [10] and protocols for ATPase measurements are given in [9].

3. RESULTS AND DISCUSSION

In the experiments described below, resonance energy transfer was measured between BrAEDANS and IAF as the donor-acceptor pair by the donor quenching technique [15]. The acceptor (IAF) was selectively reacted with Cys-374 of

actin and the donor (BrAEDANS) was located on Cys-177 of the alkali 1 (A1) light chain in S1(A1).

3.1. *Resonance energy transfer in the binary acto-S1 complex*

Fig.1a shows an example of resonance energy transfer between F-actin-AF and S1(A1-AEDANS) and the results from several such experiments are summarised in table 1. The efficiency of transfer (E) in the binary, rigor acto-S1 complex was 24 ($\pm$ 1.1)% which corresponds to a spatial separation of the fluorophores, calculated as $R(2/3)$, of 5.7 nm. When the complex was formed with the regulatory protein components, tropomyosin and troponin, E was not significantly altered in either the presence or absence of Ca^{2+} (table 1). Energy transfer in these systems confirms the ability of S1 to complex with regulated actin in the absence of Ca^{2+} as shown in earlier binding [18,19] and fluorescence [9] studies.

3.2. *Resonance energy transfer in the ternary acto-S1–AdoPP[NH]P and acto-S1–ADP complexes*

Resonance energy transfer measurements carried out on ternary complexes formed with actin-AF, S1(A1-AEDANS) and the non-hydrolysable ATP analogues, ADP and adenosine Ado*PP*[NH]*P*, using the strategy employed earlier [9], are shown in table 2. In both ternary complexes, an average transfer efficiency of 38% was consistently found, representing a small decrease of some 0.6 nm in the distance between the fluorophores (table 2). This could reflect similarities in the conformational states adopted by the acto-S1–nucleotide complexes that are distinct from that of the rigor conformation. A variety of other methods have concluded that the ternary complex formed with these nucleotides can exist in (at least) 2 conformations [20–23]. It should be emphasized, however, that this small change in distance is within the range of R generated by an analysis of k^2 ([9]; and below).

3.3. *Resonance energy transfer in the binary acto-pPDM–S1 and the ternary acto-S1–ATP complexes*

A derivative of S1(A1-AEDANS) was prepared using *p*PDM to crosslink the 2 thiol groups [7,13] and the results obtained using this S1·ATP

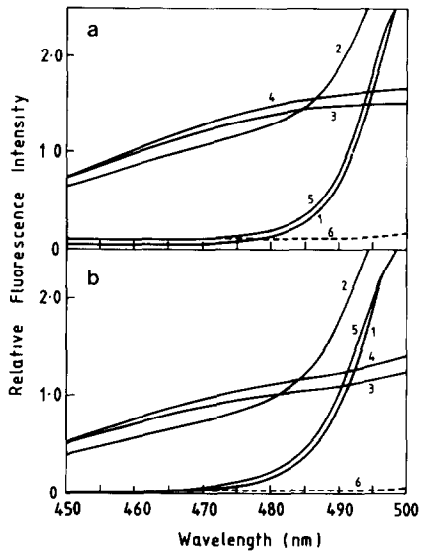

Fig.1. Corrected fluorescence emission spectra of F-actin-AF and S1(A1-AEDANS). The different scans are recordings of the fluorescence emission spectra of singly and doubly labelled acto-S1 complexes as follows: 1, F-actin-AF; 2, F-actin-AF + S1(A1-AEDANS); 3, S1(A1-AEDANS); 4, F-actin + S1(A1-AEDANS); 5, F-actin-AF + S1(A1); 6, F-actin + S1(A1). The excitation monochromator was set at 360 nm and the corrected emission spectra are shown. Donor quenching is shown by the difference between scans 4 and 2 and was monitored routinely at 468–475 nm where acceptor emission was negligible. (a) The concentration of the S1(A1) and S1(A1-AEDANS) was 2 μM and that of F-actin and F-actin AF 5 μM. Proteins were dissolved in 25 mM Tris-HCl, pH 8, containing 4 mM $MgCl_2$, 4 mM KCl, 0.25 mM dithiothreitol and 0.02% sodium azide. Under these conditions, virtually all the S1(A1) is complexed with actin. (A1-AEDANS, 1.0 mol label per mol protein; actin-AF, 0.76 mol label per mol protein.) (b) The resonance energy transfer is shown between F-actin-AF and *p*PDM-S1(A1-AEDANS). The concentration of F-actin species was 9 μM and that of S1 species 2 μM. The experiments were carried out in 10 mM imidazole-HCl, pH 7.0, containing 2 mM $MgCl_2$, 5 mM KCl and 1 mM dithiothreitol. The concentration of the binary actin-AF-S1(A1-AEDANS) transferring species under these conditions was 0.62 μM and was determined by spinning down the doubly labelled complex as described in section 2. (A1-AEDANS, 0.75 mol label per mol protein; actin-AF, 0.68 mol label per mol protein.)

Table 1

Resonance energy transfer between S1(A1-AEDANS) and F-actin-AF in the absence and presence of tropomyosin and troponin

Donor	Acceptor	Transfer efficiency, E (%)	$R(2/3)$ (nm)
S1(A1-AEDANS)	F-actin-AF	24 (± 1.1)	5.7
S1(A1-AEDANS)	F-actin-AF + tropomyosin/troponin (+ 0.2 mM $CaCl_2$ or 1 mM EGTA)	19 (± 1.3)	6.0

E values (± SE) were the average of about 20 determinations on 4 different protein preparations and did not change when (i) the concentration of KCl was varied between 4 and 150 mM, (ii) the protein concentrations were varied between 1 and 8 μM and (iii) the actin/S1 ratio was varied between 2.5 and 4. Tropomyosin-troponin was prepared as a complex [33] and mixed with actin in a molar ratio of 7:2 (actin:complex). The actin-activated Mg-ATPase activities of these labelled regulated acto-S1 complexes were at least 70% Ca-sensitive. The spectral parameters used for calculation of E, determined as in [9], were: ϕ_D, the quantum yield of the donor-labelled S1 in the absence of acceptor, 0.39; J, the spectral overlap integral of donor fluorescence and acceptor absorption, 1.868×10^{15} $nm^4 \cdot M^{-1} \cdot cm^{-1}$. $R_0(2/3)$, the critical transfer distance at which transfer efficiency is 50%, was 4.72 nm. The refractive index of the medium was assumed to be 1.4 [15] and a value of $k^2 = 2/3$ was used in these calculations

Table 2

Resonance energy transfer in ternary complexes formed from S1(A1-AEDANS), F-actin-AF and either Ado*PP*[NH]*P* or ADP

Ligand (L)	Initial concentrations			Equilibrium concentrations			E (%)	$R(2/3)$ (nm)
	A (μM)	M (μM)	L (mM)	ML (μM)	AM (μM)	AML (μM)		
Ado*PP*[NH]*P*	7.68	1.89	3.7	0.95(50)	0.04(2)	0.90(48)	38	5.1
ADP	8.38	1.89	3.7	0.94(50)	0.04(2)	0.91(48)	38	5.1

A, F-actin-AF; M, S1(A1-AEDANS); L, Ado*PP*[NH]*P* or ADP. The equilibrium concentrations of the various species were determined from the 4 binding constants describing the formation of the abortive ternary complexes for actin, S1 and either Ado*PP*[NH]*P* [20] or ADP [24] under the conditions described in tables 6 and 7 of [9]. The values in parentheses are the percentage of the initial [S1] in the various complexes. Values of E are the average of 3 determinations

analogue in resonance energy transfer experiments with F-actin-AF are described in fig.1b and table 3. As the *p*PDM-S1 binds to actin more weakly than native S1, it was necessary to determine the amount of complex present. This was carried out by spinning down the doubly labelled acto-*p*PDM–S1, as described [17], at the same time as the fluorescence measurements were made. These data were crucial to the calculation of the results and further details are given in table 3. With 3 different protein preparations and concentrations, the fluorescence of the donor fluorophore in the complex was entirely quenched (i.e., $E > 95\%$). This now represents a spatial separation of the

Table 3

Resonance energy transfer within the binary F-actin-AF–*p*PDM-S1(A1-AEDANS) complex

Initial concentrations		Concentration of transferring species (μM)	E (%)	$R(2/3)$ (nm)
F-Actin-AF (μM)	pPDM-S1-(A1-AEDANS) (μM)			
10.0	3.0	0.87	>95	<2.8
10.0	2.5	0.85	>95	<2.8
9.0	2.0	0.62	>95	<2.8

The experiments were carried out in 10 mM imidazole-HCl, pH 7.0, 0.5 mM $MgCl_2$, 1 mM dithiothreitol and 5 mM KCl at 25°C. The concentration of the transferring species was determined by spinning down samples of the reaction mixture at the same time as the fluorescence measurements were made as described in section 2. The ϕ_D and spectral characteristics of *p*PDM-S1(A1-AEDANS) were identical to the uncrosslinked hybrid

fluorophores of $R(2/3) < 2.8$ nm. In other words, the 2 fluorophores have moved closer by at least 3 nm. This is the first demonstration of a large conformational change in the acto-S1 complex, suggesting that the *p*PDM crosslinking does indeed arrest the contractile process at the pre-power stroke stage and so demonstrates the existence of a second attached state structurally distinct from that found in rigor. The addition of 4 mM ATP to these samples did not affect the results ($E > 95\%$), as anticipated if the crosslinking of the S1 thiols was complete and no rigor complex was present.

The calculation of $R(2/3)$ values quoted above assumes isotropic distribution for the transition moments of the 2 fluorophores (i.e. the orientation factor, $k^2 = 2/3$). This is rarely likely to be the case in practice [9,15], but even so it is inconceivable that changes in k^2 could totally account for the differing values of E reported in table 3. Nevertheless, an estimate of the limiting values of R can be obtained from measurement of the average depolarisation factors ($<d>$) [25]. These have already been determined for F-actin-AF in the presence of S1 [9] ($<d> = 0.491$) and for S1(A1-AEDANS) [11] ($<d> = 0.313$). From Dale et al. [25], limiting values of k^2, R_0 and hence R can be determined; for the rigor actin-AF-S1(A1-AEDANS) complex, $R_{min} = 4.9$ nm, $R_{max} = 7.0$ nm and $R_{mean} = 6.0$ nm and for actin-AF-*p*PDM-S1(A1-AEDANS) $R_{min} = 2.4$ nm, $R_{max} = 3.6$ nm and $R_{mean} = 3.2$ nm. It must be noted, however, that in the latter case these values were calculated from $E = 95\%$, which is itself an extreme value and precise R_{min} and R_{max} estimates cannot be derived, therefore R_{mean} is likely to be <3 nm.

Since the change in E was so dramatic, a single experiment was also performed in which 4 mM ATP was added directly to rigor samples of actin-AF-S1(A1-AEDANS) (i.e. none crosslinked) and the spectra collected within 1 min, when ATP hydrolysis was judged to be less than 15%. In this case, when a binding constant of 2×10^4 M^{-1} [26] for S1 · ATP (or S1 · ADP · P_i) to actin was used, E was again found to be >95%, suggesting that *p*PDM-S1 is a good analogue of the S1 · ATP and thus the pre-power stroke state.

Two further control experiments were performed to reinforce the validity of the energy transfer experiments. In the first, the addition of guanidine-HCl to 6 M to these samples caused complete loss of donor quenching. Secondly, energy transfer was monitored by sensitized emission [15] in the presence of acrylamide. This collisional quencher competes with the energy transfer process and causes a decrease in the sensitized emission. This observation excludes the possibility that the donor quenching noted in the energy

transfer experiments was the result of a conformation change induced by the chemical modification of actin with the fluorescein dye (or of crosslinking the S1 thiols) causing some non-specific long-range quenching.

The results presented here demonstrate for the first time that a considerable shift in distance (estimated extremes of 1.3 and 4.6 nm, with a mean of >3 nm) between a point on S1 and a point on actin occurs when changing from a weak to a strong binding complex. The extensive binding studies by Greene and Eisenberg [6,18,19,24,26] show that these 2 states represent the pre- and post-power stroke intermediates often designated as myosin head moving from a 90 to a 45° attitude to the axis of the actin filament [19]. This study is consistent with such a change in orientation as being the basis of the power stroke and therefore the energy transduction step. Further support for these observations comes from ^{1}H-NMR studies on the acto-*p*PDM–S1 complex [27], where it was noted that the *p*PDM-S1 binds to actin without the extensive loss of side chain mobility in the CH_3, CH_2 envelope associated with the formation of either the rigor acto-S1 complex [28] or the ternary acto-S1–Ado*PP*[NH]*P* complex [9]. This 'flexible' binding of *p*PDM-S1 to actin provides additional structural data consistent with the fluorescence results in identifying a second attached state of the actomyosin complex.

The only other sites that can be specifically labelled on actin and S1 that appear close enough for resonance energy transfer studies are the heavy chain SH_1-thiol on S1 and Cys-374 on actin [9,16]. In an extensive study using donor-acceptor pairs at either the ATPase or SH_1-thiol of S1 and either the nucleotide-binding site or Cys-374 of actin, Dos Remedios and Cooke [29] were unable to find significant energy transfer between any other sites in the rigor complex, thus setting some limits on the position of the myosin active site and the SH_1-thiol relative to actin. In light of the present study it would be interesting to repeat some of these experiments using the *p*PDM-S1 derivatives.

It is still possible to reconcile these data with the spin labelling experiments [5] if it is assumed that the probed region of S1 in our experiments can move whereas the domain containing the spin label (on the SH_1 thiol) remains rigidly orientated to actin. Nevertheless, the resonance energy transfer experiments are consistent with there being enough information within the myosin head to account for the energy transduction step [30] without invoking another part of the molecule. This report does not, of course, resolve how much of the change in distance occurs within the S1 as opposed to between actin and S1 as a result of some reorientation. It is likely that both occur, with some rejuxtapositioning of the myosin domains, as a result of ATP binding/hydrolysis, causing a reorientation of the S1 relative to the actin filament. Such movements within monomeric kinases as a consequence of substrate binding have been readily observed [31,32].

ACKNOWLEDGEMENTS

The authors thank the MRC and the SERC for financial support. We are also grateful to Dr R.E. Dale for discussions and helpful suggestions during the course of this work.

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