Polarization Microfluorimetry Study of Interaction Between Myosin Head and F-Actin in Muscle Fibers

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Abstract. Changes in conformation of F-actin induced by the binding of myosin molecule subfragment 1 were studied in myosin-free single ghost muscle fibers with the method of polarization microfluorimetry. The modification of the structure of subfragment 1 by proteolytic digestion with one or two cuts in subfragment 1 or degradation of 50 kDa domain did not influence the character of changes in the conformation of F-actin. The use of preparations of subfragment 1 devoid of the 20 kDa domain or both cross-linked SH_1 and SH_2-groups changed the character of conformational rearrangements in F-actin. The present data show that a site of interaction with actin in the 20 kDa domain plays a key role in inducing the changes in actin conformation corresponding to a "strong" form of the binding. It is supposed that transmission of changes in the conformation of the myosin head to F-actin might be important for muscle contraction.

Key words: Ghost fibre — Polarized fluorescence — Conformational changes in F-actin — Myosin subfragment 1 — Muscle contraction

Introduction

The interaction between myosin, actin and ATP is the basis of muscle contraction. Investigation of conformational changes of these contractile proteins appears to be one essential stage for the understanding of the molecular mechanism of contraction.

Numerous experimental data are available on conformational changes that occur in myosin during ATP hydrolysis and interaction with actin. Using fluorescent probes attached to the myosin head it has been shown that the spatial orientation of this molecule depends on the functional state of the muscle fiber (Nihei et al. 1974; Highsmith and Cooke 1983; Yanagida 1985).

Measurements of intrinsic tryptophan fluorescence polarization of actin and of

fluorescence of external dyes specifically attached to actin raised the possibility that myosin head binding induces the conformational changes in F-actin and changes in filament flexibility and actin monomer orientation (Borovikov et al. 1974, 1982a; Yanagida and Oosawa 1978; Borovikov and Chernogriadskaya 1979; Oosawa 1983). Recent data on resonance energy transfer show that myosin head binding is associated with changes of the efficiency of this transfer between Cys-374 and Lys-61 residues in actin (Miki et al. 1987).

Conformational changes in F-actin filaments appear to be dependent on calcium concentration and on the degree of phosphorylation of the myosin regulatory light chain (Borovikov et al. 1982b; Borovikov and Levitsky 1985; Szczęsna et al. 1987; Kąkol et al. 1987). Conformational changes of actin have been observed during the development of muscle fiber tension and during the transition from relaxation to contraction and to rigor state (Prochniewics-Nakajama et al. 1983; Galązkiewicz et al. 1987; Borovikov and Lebedeva 1987).

The head of the myosin molecule, subfragment 1 (S1) has been found to consist of functional domains, successively linked proteolytic fragments with molecular masses of 27, 50, and 20 kDa (Mornet et al. 1979, 1981). The actin-myosin interface involved sites within the 50 and 20 kDa fragments. The 27 and 50 kDa fragments contribute to the formation of the nucleotide binding site (Mahmood and Yount 1984).

Detailed studies of the domain structure and, in particular, of the communication between actin and the ATP binding sites appeared to be very useful for the understanding of the force generation mechanism in muscle. It has been postulated that energy transduction in the myosin molecule is based on structural communication between actin and the ATP binding sites (Audemard et al. 1988; Botts et al. 1989). Conformational changes in the ATP binding site at different stages of ATP hydrolysis are supposed to be transmitted along the heavy chain of S1 to the actin site and to determine the S1 position with respect to the actin filament.

Furthermore, it is easy to imagine that the position of S1 with respect to the actin filament determines the structural state of the actin filament. As mentioned previously, changes in the structural state of actin filaments can be revealed with the polarization microfluorimetry method (Kąkol et al. 1987). We used this method in the present experiments to study the effect of conformational changes in S1 induced by proteolysis under different conditions (Mornet et al. 1979, 1981; Burke and Sivaramakrishnan 1986), of cross-linking of SH_1 and SH_2-groups with pPDM (Wells and Yount 1982), and of the removal of the 20 kDa domain (Okamoto and Sekine 1987) on structural changes in actin filaments appearing up on the interaction with S1.

To monitor this interaction, we studied S1 binding-induced conformational changes mentioned above in ghost (myosin-free) muscle fibers. Polarization microfluorimetry of a complex of actin filaments with phalloidin-rhodamine or 1,5-

IAEDANS or S1-1,5-IAEDANS was used to detect conformational changes in the model system. These conformational changes in actin filaments and myosin heads were compared to the respective changes observed in glycerinated muscle fibers upon isometric tension development. A preliminary report of the results was published elsewhere (Borovikov et al. 1988, 1989).

Materials and Methods

Subfragment-1 and its derivatives

Subfragment-1 was prepared from rabbit skeletal muscle myosin (Ivanov and Juriev 1961) with N-tosyl-L-lysine chloromethyl ketone, treated by α-chymotrypsin digestion for 10 min at 25 °C in the absence of Ca^{2+} (Wagner and Weeds 1977), and purified by gel filtration on a Sephacryl S-200 column (2.6 × 90 cm).

To prepare S1 nicked at junctions between fragments 27, 50 and 20 kDa ($S1_t$) and between fragments 27 and 70 kDa, ($S1_{a/t}$) S1 was digested with trypsin - treated N-tosyl-L-phenylalanine chloromethyl ketone (TPCK-trypsin) at an enzyme to protein ratio of 1:50 (w/w) for 30 min at 25 °C in the absence and presence of actin, respectively (Mornet et al. 1979).

To prepare S1 without the 50 kDa domain, ($S1_{m/t}$) S1 was digested with TPCK-trypsin after preincubation in 20% methanol for 30 min at 25 °C (Burke and Sivaramakrishnan 1986), and purified by gel filtration on a Sephadex G-150 (fine) column (1.6 × 90 cm).

To prepare S1 without the 20 kDa domain or S'1 (75), the method of Y. Okamoto and T. Sekine was used (Okamoto and Sekine 1987). Myosin filaments were digested with TPCK-trypsin at 4 °C in the presence of 2 mmol/l $MgCl_2$ and then incubated with Mg^{2+}-ATP at 37 °C and centrifuged and purified by gel filtration on a Sephadex G-150 (fine) column (1.6 × 90 cm).

Cross-linking of the SH-groups SH_1 and SH_2 in S1 with N,N-phenylenedimaleimide (pPDM) was performed in the presence of Mg^{2+}-ADP at 0 °C (Borovikov et al. 1986).

The subunit composition of these preparations was evaluated by SDS-electrophoresis according to Laemmli (1970). The protein concentration was determined with the microbiuret method (Itzhaki and Gill 1964).

S1 and its derivatives were labeled with 1,5-IAEDANS at a reagent to protein molar ratio of 1:1 (the mass of S1 was assumed to be 110 kDa) for 24 hours at 0 °C (Margossian and Lowey 1982). The unreacted dye was separated by dialysis against 1 mmol/l $MgCl_2$, 25 mmol/l Tris-HCl buffer, pH 7.5.

The preparations were characterized by determining their Ca^{2+}-ATPase and actin-activated Mg^{2+}-ATPase activities. The incubating medium for Ca^{2+}-ATPase was the following: 0.005 mol/l ATP, 0.25 mol/l KCl, 0.01 mol/l $CaCl_2$, 0.1 mol/l Tris-HCl, pH 7.5, 0.1 mg/ml protein; for Mg^{2+}-ATPase: 0.005 mol/l ATP, 0.006 mol/l KCl, 0.004 mol/l $MgCl_2$, 0.06 mol/l Tris-HCl, pH 7.5, 0.1 mg/ml S1 and 0.4 mg/ml F-actin, incubation time was 15 min at 37 °C. The reaction was terminated by addition of TCA at 4% final concentration. P_i was determined according to Fiske and Subbarow (1925).

Glycerinated and ghost muscle fibers

Single glycerinated muscle fibers from rabbit m.psoas prepared as described elsewhere (Borovikov and Gusev 1983), were washed with a solution containing 0.1 mol/l KCl, 0.001

mol/l $MgCl_2$, 0.067 mol/l phosphate buffer, pH 7.0 (standard solution) to remove glycerol. Then, the contractile proteins in the fibers were modified with fluorescent labels: in some fibers F-actin was labeled with phalloidin-rhodamine, in others with 1,5-IAEDANS. The relaxing solution contained 10 mmol/l KCl, 5 mmol/l $MgCl_2$, 5 mmol/l ATP, 5 mmol/l EGTA, 6.7 mmol/l phosphate buffer, pH 7.0; for contraction, 4.5 mmol/l $CaCl_2$ were added to the relaxing solution.

Ghost fibers were prepared by extracting single muscle fibers with a solution containing 0.8 mol/l KCl, 0.01 mol/l $MgCl_2$, 0.01mol/l ATP and 0.067 mol/l phosphate buffer, pH 7.0, for 80 min at 4 °C to remove myosin, tropomyosin and troponin. The protein content in the fibers was analysed by SDS-electrophoresis (Laemmli 1970). Actin constituted approx. 80% of total protein of the ghost fibers. Before an experiment, every ghost fiber was checked for coincidence of its fluorescence anisotropy parameters with those of a thin intact fiber (Borovikov and Gusev 1983). F-actin in single ghost muscle fibers was modified by phalloidin-rhodamine and 1,5-IAEDANS. The former was added to the fiber at 40 μmol/l in standard buffer. After 30 min incubation at 0 °C the unbound reagent was washed out with standard buffer (Kąkol et al. 1987).

The labeling with 1,5-IAEDANS was performed by incubating the fibers with 100 μmol/l of the reagent in 60 mmol/l KCl, 1mmol/l $MgCl_2$, 25 mmol/l Tris-HCl, pH 8.0 for an hour at 4 °C; the unbound reagent was subsequently washed out with standard solution.

Under these conditions the majority of actin monomers became labeled with the dye. The specificity of the staining was checked with SDS-electrophoresis and by identification of fluorescence bands. The only fluorescence detected was that of actin.

Attachment to actin in the ghost fiber of subfragment 1 and its derivatives

Fibers modified with the fluorescent labels were incubated for 1–1.5 h (Borovikov and Gusev 1983) in a solution containing 1–3 mg/ml S1, 1 mmol/l $MgCl_2$, 25 mmol/l Tris-HCl, pH 7.5 at 18 °C. The exposure time for S'1 (75) and pPDM-S1 was 4 h.

To attach S1 labeled by 1,5-IAEDANS, unmodified fibers were used. Unbound S1 was removed by wash out for 15–20 min with a solution containing 10 mmol/l KCl and 6.7 mmol/l phosphate buffer, pH 7.0. The molar ratio of S1 and its derivatives to actin was checked by SDS-electrophoresis and subsequent densitometry (Laemmli 1970).

Measurements of polarized fluorescence of phalloidin-rhodamine and 1,5-IAEDANS

Fluorescence of phalloidin-rhodamine bound to F-actin was excited at 486±5 nm and monitored at 500–600 nm. When S1 or F-actin were modified with 1,5-IAEDANS the excitation and emission lines were at 365±5 nm and 480–600 nm, respectively. The measurements were carried out with a microfluorimeter (Ioffe et al. 1974).

Four intensities of polarized fluorescence were recorded: ${}_{\parallel}I_{\parallel}$, ${}_{\parallel}I_{\perp}$, ${}_{\perp}I_{\perp}$, ${}_{\perp}I_{\parallel}$, where the subscripts on the left side denote the direction of the excitation and those on the right side the direction of the emission light relative to the fiber axis. The extent of polarization was calculated as follows:

$$P_{\parallel} = ({}_{\parallel}I_{\parallel} - {}_{\parallel}I_{\perp})/({}_{\parallel}I_{\parallel} + {}_{\parallel}I_{\perp})$$
$$P_{\perp} = ({}_{\perp}I_{\perp} - {}_{\perp}I_{\parallel})/({}_{\perp}I_{\perp} + {}_{\perp}I_{\parallel})$$

Experimental data were analyzed according to the available mathematical models (Rosanov et al. 1971; Tregear and Mendelson 1975; Yanagida and Oosawa 1978; Wilson

and Mendelson 1983; Morales 1984; Kąkol et al. 1987): polarized fluorescence of a muscle fiber may be described as a system of fluorophores distributed either disorderly or along a spiral in the surface of a cone, the axis of which coincides with that of the muscle fiber. Analyzing the data it was assumed that the actin filament is not rigid (Yanagida and Oosawa 1978; Oosawa 1983; Kąkol et al. 1987) and that the angle between the fiber axis and the filament is different from zero. The four intensities of polarized fluorescence can be expressed as functions of angles Φ_A, Φ_E, Θ and the value N, where Φ_A and Φ_E are angles between the long axis of F-actin filament and the adsorption and emission dipoles, respectively; Θ is the angle between the F-actin filament long axis and the fiber axis; and N is the relative number of fluorophores distributed in a disordered fashion. The corresponding equations were described elsewhere (Kąkol et al. 1987). Angle γ, or difference between angles Φ_A and Φ_E, was used for calculations. This angle has a constant value for every fluorophore in the protein molecule (Tregear and Mendelson 1975). To defect changes in the microenvironment of a fluorescent label in actin upon the interaction of S1 with actin filaments we studied emission spectra of fluorescence of phalloidin-rhodamine and 1,5-IAEDANS bound to actin and acto-S1 complex. No changes in maximum positions of the spectra were revealed. This speaks against substantial changes in the label microenvironment.

Results

Characterization of S1 and its derivatives

Unmodified S1 and S1 digested with trypsin under different conditions were used to attach to ghost muscle fiber actin. Typical ATPase activities of these preparations are listed in Table 1. Electrophoreograms of these preparations are shown in Fig. 1*A*.

As can be seen from Fig. 1*A*, preparation of $S1_t$ obtained by 10 min digestion with trypsin contained three heavy chain fragments of 25, 50 and 20 kDa corresponding to fragments obtained by other authors (Mornet et al. 1979, 1981). Trypsin digestion in the presence of actin yielded a preparation of $S1_{a/t}$ containing 25 and 70 kDa fragments.

The ATPase activities of these preparations (Table 1) are comparable to those described earlier (Mornet et al. 1979, 1981). Cleavage of the heavy chain at the junction between the N-terminal 25 kDa fragment and the 70 kDa fragment did not impair the Ca^{2+}-activated ATPase and the actin-activated Mg^{2+}-ATPase of original S1; when the heavy chain was cleaved at both the 25 kDa/50 kDa and the C-terminal 20 kDa/50 kDa junction, the extent of actin activation of the Mg^{2+}-ATPase decreases by a factor of 3.

It is known that the tryptic fragments of S1 are hold together by noncovalent forces (Mornet et al. 1979, 1981). To separate them it is necessary to apply denaturing agents such as 6 mol/l guanidine hydrochloride or 0.1% sodium dodecylsulphate (Mühlrad and Morales 1984; Okamoto and Sekine 1987; Mühlrad 1989). After removal of the denaturing agents, the 50 and 20 kDa fragments can

Table 1. The effect of limited proteolysis on ATPase activity of S1

Preparation	ATPase activity in the presence of			Actin activation of Mg^{2+}-ATPase
	Ca^{2+}	Mg^{2+}	Mg^{2+} and actin[a]	
	μmoles × min^{-1} × (mg protein)$^{-1}$			-fold
S1	1.86	0.024	0.35	15
$S1_t$	2.06	0.051	0.15	3
$S1_{a/t}$	1.51	0.012	0.17	14
$S1_{m/t}$	0	0	0	–
$S1+S1_{m/t}(1:8)$[b]	–	0.024	0.10	4
$S1+S1_{m/t}(1:40)$[b]	–	0.024	0.05	2
S'1(75)	0.5	0.02	0.02	1

[a] Actin: S1 molar ratio was 10:1

[b] Molar ratio $S1:S1_{m/t}$

S1 derivatives: $S1_t$; $S1_{a/t}$; $S1_{m/t}$; S'1(75) - treated with trypsin; the same in the presence of actin; the same in the presence of methanol; devoid of the 20 kDa domain, respectively.

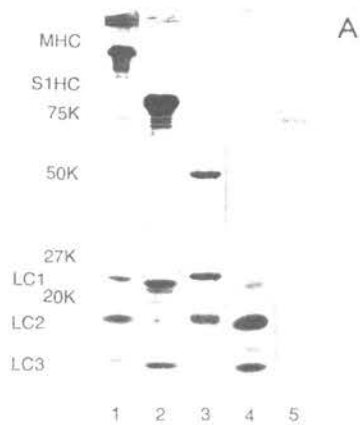

Figure 1 A. SDS 12% polyacrylamide gel electrophoresis of S1 and its derivatives. (1) myosin, (2) subfragment-1, (3) subfragment-1 treated with trypsin, (4) subfragment-1 treated with trypsin and 20% methanol, (5) subfragment-1'(75) according to Okamoto and Sekine (1987)
Abbreviations: MHC - myosin heavy chain; S1HC - S1 heavy chain; α - α-actinin; A - actin: LC1, LC2, LC3 - myosin light chains. For conditions, see Materials and Methods.

be renatured.

In the 50 kDa fragment some conformational changes can be induced by 20% methanol, and after this pretreatment trypsin cleaves this fragment into small pep-

Table 2. The effect of S1 and its derivatives on polarized fluorescence parameters of phalloidin-rhodamine attached to ghost fiber actin.

The degrees of fluorescence polarization of $P_{\parallel}$ and $P_{\perp}$ were defined as: $P_{\parallel} = ({}_{\parallel}I_{\parallel} - {}_{\parallel}I_{\perp})/({}_{\parallel}I_{\parallel} + {}_{\parallel}I_{\perp})$, $P_{\perp} = ({}_{\perp}I_{\perp} - {}_{\perp}I_{\parallel})/({}_{\perp}I_{\perp} + {}_{\perp}I_{\parallel})$. The angle of emission (Φ_E) dipole of fluorophore relative to the F-actin axis as well as $\sin^2\Theta$, (Θ is the average angle between F-actin and the fiber axis) were calculated according to Kąkol et al. 1987.

Average values and standard errors were calculated from 50–100 measurements. For the preparation of fibres, S1 and its derivatives and the conditions of experiments, see Materials and Methods. Abbreviations: F-actin - S1, complex of actin and S1; pPDM-S1, S1 treated with N, N'-phenylenedimaleimide; for other S1 derivatives see Table 1.

Preparation	$P_{\parallel}$	$P_{\perp}$	Φ_E	$\sin^2\Theta$
F-actin	0.454±0.002	-0.270±0.002	42.0±0.1	0.057±0.001
F-actin-S1	0.493±0.003	-0.309±0.002	40.5±0.1	0.061±0.001
F-actin-$S1_{a/t}$	0.496±0.003	-0.292±0.003	40.3±0.1	0.071±0.001
F-actin-$S1_t$	0.502±0.002	-0.295±0.001	40.1±0.1	0.073±0.001
F-actin-$S1_{m/t}$	0.498±0.003	-0.308±0.002	40.0±0.1	0.074±0.001
F-actin-S'1(75)	0.434±0.003	-0.259±0.002	42.6±0.1	0.054±0.001
F-actin-pPDM-S1	0.441±0.003	-0.263±0.002	42.3±0.1	0.053±0.001

tides (Burke and Sivaramakrishnan 1986). So, the complex of 20 and 25 kDa fragments together with the light chains leaves, after methanol treatment and trypsin digestion, $S1_{m/t}$ (Fig. 1A). It has no ATPase activity, but competes with native S1 for actin binding and even decreases actin activation of S1 Mg^{2+}-ATPase (Table 1).

Table 1 also shows the results of measurements of the ATPase activity of the N-terminal 75 kDa fragment of S1 heavy chain. (This fragment, first prepared by Okamoto and Sekine (1987), is devoid of the 20 kDa domain (Fig. 1*A*)). Our preparation of S'1 (75) showed some Ca^{2+}-activated ATPase activity (Table 1), but in contrast to Okamoto and Sekine, we were not able to find any actin-activated Mg^{2+}-ATPase activity in this preparation.

Effects of S1 and its derivatives on polarized fluorescence parameters of phalloidin-rhodamine labelled actin

Ghost muscle fibers with the actin modified by phalloidin-rhodamine were incubated with the mentioned preparations of S1 and its derivatives. The electrophoreograms in Fig. 1*B* show that under the conditions of the experiment (cf. "Materials and Methods") these preparations attached to ghost fiber F-actin.

As can be seen from Table 2, S1 binding to the fibers results in an increase in the absolute values of $P_{\parallel}$ and $P_{\perp}$; this is not the case for S1'(75) and pPDM-S1.

Computer analysis of the data shows that uncoupling of the domain at one ($S1_{a/t}$) or two junctions ($S1_t$), or removal of the 50 kDa domain ($S1_{m/t}$), result in a reduction of angle Φ_E and an increase of $\sin^2\Theta$ (Table 2). The increase of $\sin^2\Theta$ is indicative of a higher flexibility of thin filaments, whereas the reduction of Φ_E

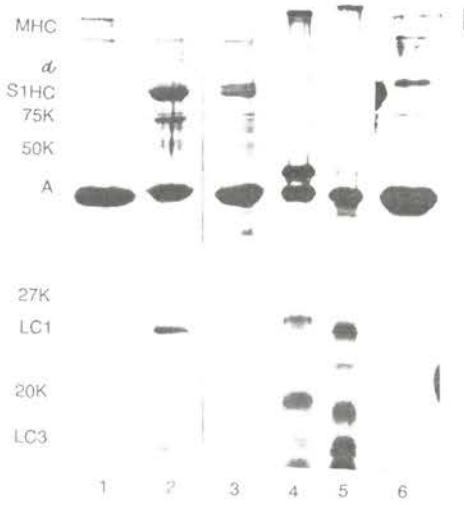

Figure 1B. SDS 12% polyacrylamide gel electrophoresis of S1 and single ghost muscle fibers.
(1) ghost muscle fiber before and (2) after attachment of subfragment-1, (3) p-PDM treated S1, (4) subfragment-1 treated with trypsin, (5) subfragment-1 treated with trypsin and 20% methanol, (6) subfragment-1'(75). Abbreviations see Fig. 1A.

reflects changes in the orientation of the respective areas of actin monomers in thin filaments (Yanagida and Oosawa 1978; Kąkol et al. 1987); thus the attachment of the mentioned derivatives of S1 does not change the nature of conformational rearrangements of actin observed with native S1. With nicked S1 a higher flexibility of thin filaments was observed as $\sin^2 \Theta$ increased from 0.061 to 0.074 (Table 2) in the sequence S1, $S1_{a/t}$, $S1_{m/t}$. This appears to mean that changes in S1 conformation are transmitted to some areas of actin monomer.

Table 2 also shows data concerning the effect of S1 modified by pPDM (pPDM-S1). The treatment of S1 with this agens in the presence of nucleotide leads to "locking" of Mg^{2+}-ADP in the myosin active site (Wells and Yount 1982). Such a complex is a stable analog of the ATPase cycle intermediates M*-ATP and M**-ADP-P_i (Chalovich et al. 1983). It has no ATPase activity. Nevertheless, it modified S1 attached to F-actin of ghost fibers, though to lesser extent than did other preparations of S1 (cf. Fig. 1*B*).

This preparation induced only small changes in $P_\parallel$ and $P_\perp$ values, and it slightly increased the angle Φ_E and decreased $\sin^2 \Theta$ (Table 2). These results resemble those obtained with S'1 (75) which is devoid of the 20 kDa domain (Table 2). Based on earlier data concerning the effect of SH_1-SH_2 crosslinking in subfragment 1 (Borovikov et al. 1986) on tryptophan fluorescence anisotropy of actin filament

with attached S1, it has been concluded that the changes in actin filaments in this case differ from those occurring upon S1 binding. This difference has been considered to be a result of "weak" binding of S1 in complex with ATP or ADP-P_i and "strong" binding of S1 without the nucleotide (Wells and Yount 1982; Chalovich et al. 1983).

Since the S'1 (75) preparation is able to bind actin (Fig. 1*B*), the similarity of its effect on polarized fluorescence of phalloidin-rhodamine attached to ghost fiber F-actin to that of pPDM-S1 is consistent with its "weak" binding, and indicated that the 20 kDa domain plays a key role in inducing the changes in actin conformation. This is supported by the results obtained with $S1_{m/t}$ devoid of the 50 kDa domain (Fig. 1*A*, Table 2). Although this preparation lacked ATPase activity, it was bound to F-actin and induced in it changes analogous to those induced by native S1 ("strong" binding).

According to earlier interpretation the cross-linking of SH_1-SH_2 groups in myosin heads substantially changes their interactions with actin so that the modified heads lose the ability to "switch on" actin filaments at binding in the absence of regulatory light chains (Borovikov and Levitsky 1985). Analogically it may be supposed that "the switching on" is weakened or absent upon removal of the 20kDa domain: according to our earlier results the "on"-state is characterized by reduced Φ_E and an increased value of $\sin^2\Theta$ (Kąkol et al. 1987). According to this interpretation, domain uncoupling increases the number of actin monomers in the "switched on" state (cf. gradual reduction of Φ_E and increase of $\sin^2\Theta$ with $S1_{a/t}$, $S1_t$, $S1_{m/t}$, Table 2).

Effects of S1 and its derivatives on polarized fluorescence parameters of actin labeled with 1,5-IAEDANS

According to recent concepts concerning the structure of actin, its monomer consists of two domains, one "large" and one "small", each of them containing two subdomains (Kabsch et al. 1990). At filament formation the large domain is at small radius and the small domain is at large radius of the thin filament (Holmes et al. 1990). The large domains form the core of the filament, the small domains are outer part of the filament and link with the rest of the molecule not hardly (Erickson 1989).

The fluorescent label of phalloidin-rhodamine is distributed between the large and the small domains of actin and predominantly binds to the large domain (Vandekerckhove et al. 1985; Barden 1987). 1,5-IAEDANS used in the present experiments binds to Cys-374 within the small domain. Cys-374 modification has been used in numerous investigations, and interaction with S1 results in a change of the structure of actin in the vicinity of this group (Borovikov et al. 1988; Mossakowska et al. 1988).

As can be seen from data presented in Table 3, polarized fluorescence of 1,5-

Table 3. The effect of S1 and its derivatives on polarized fluorescence parameters of 1,5-IAEDANS attached to ghost muscle fiber actin.

The values of parameters $P_{\|}$, $P_{\perp}$, Φ_E, $\sin^2\Theta$ and N were calculated as described in the legend to Table 2. For symbols and the conditions, see Table 2.

Preparation	Parameters of polarized fluorescence			
	$P_{\|}$	$P_{\perp}$	Φ_E	N
	(relative units)		(°)(relative units)	
F-actin	0.164±0.002	0.092±0.001	53.7±0.1	0.326±0.009
F-actin-S1	0.060±0.003	0.132±0.002	56.6±0.1	0.082±0.008
F-actin-$S1_{m/t}$	0.110±0.004	0.134±0.001	57.0±0.1	0.039±0.011
F-actin-S1'(75)	0.159±0.003	0.105±0.002	53.2±0.1	0.393±0.009
F-actin-pPDM-S1	0.184±0.003	0.152±0.002	53.3±0.1	0.491±0.011

IAEDANS bound to Cys-374 significantly changes upon the attachment to F-actin of S1 or $S1_{m/t}$: $P_{\perp}$ increases and $P_{\|}$ decreases. A computer analysis showed the value of Φ_E to increase and that of N to decrease both with $S1_{m/t}$ and with original S1. This supports our suggestion that removal of the 50 kDa domain does not change the rearrangement pattern in F-actin fiber upon S1 attachment. Additional support for the importance of the 20 kDa domain follows from experiments with S'1 (75) and pPDM-S1: the value of $P_{\perp}$ increases or remains unchanged; $P_{\|}$ increases; calculated parameters Φ_E and N change in the direction opposite to that observed upon the attachment of native S1; Φ_E slightly decreases, N increases (Table 3).

A comparison of changes of Φ_E of fluorophores phalloidin-rhodamine and of 1,5-IAEDANS (Table 3) shows that they move in opposite direction: for phalloidin-rhodamine the value of Φ_E decreases, whereas it increases for 1,5-IAEDANS. As the labels used bind to different areas of actin, we suppose that some parts of the polypeptide chain may move upon S1 attachment. We tried to imagine that the difference between these two values of $\Phi_E(\beta_1)$ is an indicator of a linear relation between these two points of the actin molecule. The values of β_1 in Fig. 2*A* are angles between the directions of flourescence dipoles of phalloidin-rhodamine and 1,5-IAEDANS in actin filaments. The value of β_1 increases from 11.7° to 16.0–17.0° upon the attachment of S1 and $S1_{m/t}$, and it decreases to 10.6–11.0° with S1'(75) and pPDM-S1. As it can be seen from Tables 2 and 3, this difference corresponds to the opposite direction of changes in motility of the labels.

Likely, derivatives of S1 with blocked SH_1 and SH_2 or with the 20 kDa domain removed, bind "weakly" to actin, and this is a model for complex formation of actomyosin with products of ATP hydrolysis. As can be seen from Fig. 2*A*, in this case the change in the position of the regions of the actin molecule differs from that seen with the rigor complex. Taken together, we see a typical pattern of changes in the actin filament motility (Tables 2,3) which may be a consequence of "weak" binding.

Table 4. The effect of limited proteolysis of S1 on polarized fluorescence parameters of 1,5-IAEDANS attached to S1 in acto-S1 complex of ghost muscle fibers.
The values of parameters $P_{\parallel}$, $P_{\perp}$, Φ_E and N were calculated as described in the legend to Table 2. Average values and standard errors were calculated from 60 measurements. For symbols, see Table 2.

Preparation	$P_{\parallel}$	$P_{\perp}$	Φ_E	N
	(relative units)		(°)	(relative units)
F-actin-S1	0.318±0.003	-0.202±0.002	45.6±0.1	0.084±0.002
F-actin-$S1_{a/t}$	0.288±0.004	-0.187±0.004	46.5±0.1	0.060±0.002
F-actin-$S1_t$	0.313±0.003	-0.213±0.002	46.7±0.1	0.055±0.001
F-actin-$S1_{m/t}$	0.262±0.006	-0.105±0.002	51.0±0.1	0.120±0.003

It can be assumed that there is some correlation between the different stages of the interaction of ATP with the actomyosin complex and the structural state of some areas in actin monomer.

With the SH_1-group in the 20 kDa domain of S1 labeled with 1,5-IAEDANS it is possible to distinguish changes in S1 corresponding to marked changes in the structure of actin.

Effect of S1 modification on polarized fluorescence parameters of the S1-F-actin complex labeled with 1,5-IAEDANS

We can see from Table 4 that S1 attachment to ghost muscle fiber F-actin results in the appearance of fluorescence anisotropy of 1,5-IAEDANS bound to the SH_1-group of the myosin head. This suggests an ordered displacement of the corresponding regions of myosin. According to accepted models (Rosanov et al. 1971; Tregear and Mendelson 1975; Morales 1984) this ordered displacement can be characterized by the angle of orientation of dipoles of emission fluorophores with respect to the actin filament axis. Upon trypsin digestion of S1 in one ($S1_{a/t}$) or two junctions ($S1_t$), the orientation and motility of the label bound to SH_1 changes, angle Φ_E increases and the fraction of disorderly situated fluorophores (N) decreases (Table 4).

Our results agree with the available data: proteolysis of S1 is known to raise the content of α-helix by 12% (Chaussepied et al. 1986), certain areas of S1 become more mobile (Gooddearl et al. 1985; Highsmith and Eden 1987). Also, it has been shown that the angular distribution of fluorophores of 1,5-IAEDANS in S1 attached to muscle fibers differed in rigor for split S1 as compared with native S1 (Ajtai and Burghardt 1987).

Much greater changes of values of Φ_E and N were observed with S1 with the 50 kDa domain removed by trypsinolysis in the presence of methanol (Table 4): Φ_E increased by 5.4° and N increased by 0.036. Thus, the removal of the 50 kDa domain increases the motility of the label bound to the SH_1-group of the 20 kDa domain.

Considering the angle between the direction of dipoles of phalloidin-rhodamine fluorophores in actin and 1,5-IAEDANS in the 20 kDa domain as an indicator of their respective orientation (Fig. 2*B*), we see a slight increase of this angle β_2 with derivatives of $S1_{a/t}$ and $S1_t$ and a more than twofold increase with $S1_{m/t}$. This large change in $S1_{m/t}$ orientation corresponds to the change in the fluorophore orientation at Cys 374 in the small domain of actin (β_1), and can be considered as being a result of a coordination of movements of the 20 kDa domain and the small domain of actin. This assumption finds support in our recent data on the coordinated movements of these areas of the proteins upon the addition of Mg^{2+}-ADP (Borovikov et al. 1990). Moreover, this may mean that the changes in C-terminal part of actin monomer (Seidel 1980) are a consequence of changes in orientation of the 20 kDa domain.

As the changes of β_2 with $S1_{m/t}$ are much larger than those with $S1_{a/t}$ and $S1_t$, a stabilizing effect of the 50 kDa domain on the flexibility of the 20 kDa domain may be suggested.

The facts presented herein suggest conformational changes to occur in C-terminal domain upon proteolytic digestion of the myosin head. If this domain is intact, corresponding changes occur in F-actin filaments which bind to the myosin heads. Induced the proteolysis - induced conformational changes of the head are transmitted to actin. However, is there some conformity between this change and the above - mentioned changes in actin structure observed during muscle contraction? The method of polarizated fluorimetry allows to compare changes in ghost muscle fibers at rigor complex formation with those occurring in glycerinated muscle fibers during isometric contraction.

In experiments with glycerinated muscle fibers, actin filaments were labeled with phalloidin-rhodamine, and myosin heads were labeled with 1,5-IAEDANS (Table 5). In relaxed state, fluorescence anisotropy was observed in both the actin the myosin filaments suggesting an ordered arrangement of both labels. During contraction, changes in the degrees of polarization occurred between $P_{\parallel}$ and $P_{\perp}$ with the exception of the label bound to myosin. The calculated changes in angles of dipoles of emission fluorophores with respect to the fiber axis show a decrease of Φ_E by 1.6° for phalloidin-rhodamine bound to actin and increase of Φ_E by 1.6° for 1,5-IAEDANS.

Considering the difference in angles Φ_E between the labels in myosin and actin as a measure of the respective distribution of the labels and the corresponding areas of myosin and actin, an increase from 6.6° to 9.8° is noted upon the transition from relaxation to contraction (Fig. 2*C*). Thus, the extent of the change corresponds to that observed upon the formation of rigor complex with $S1_{m/t}$ (Fig. 2*B*).

Upon isometric contraction, a substantial increase of F-actin filament flexibility occurred as evidenced by the increase of $\sin^2 \Theta$. Under the same conditions, the flexibility of myosin heads as well as that of actin monomer decreased (*N* di-

Table 5. The effects of isometric tension development on polarized fluorescence parameters of phalloidin-rhodamine and 1,5-IAEDANS bound to F-actin and myosin. For symboles, see Table 2.

Fiber state	$P_{\parallel}$	$P_{\perp}$	Φ_E	$\sin^2\Theta$	N
			Actin-linked fluorescence		
Relaxation	0.484±0.003	-0.399±0.002	40.4±0.1	0.40±0.001	–
Contraction	0.519±0.001	-0.340±0.002	38.8±0.3	0.061±0.001	–
			Myosin-linked fluorescence		
Relaxation	0.271±0.002	-0.008±0.001	47.0±0.1	–	0.298±0.005
Contraction	0.273±0.003	-0.078±0.002	48.6±0.3	–	0.183±0.003

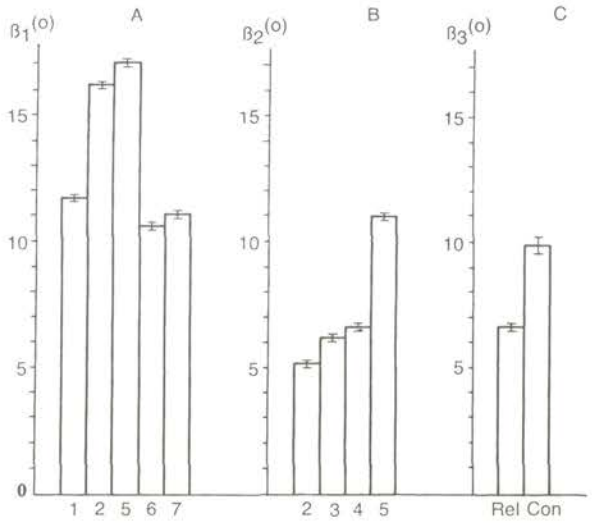

Figure 2. Effects of S1 and its derivatives on angle between two fluorescent dipoles in actin filaments - S1 complex. A: β_1 is the angle between phalloidin-rhodamin and 1,5-IAEDANS bound to actin filaments; B: β_2 is the angle between phalloidin-rhodamin bound to actin filaments and 1,5-IAEDANS bound to SH_1 in the 20 kDa domain; C: β_3 is the angle between phalloidin-rhodamin bound to actin filaments and 1,5-IAEDANS bound to myosin filaments of glycerinated muscle fibers. The same conditions as given in Tables 2–4.
Abbreviations: 1, F-actin; 2, F-actin-S1; 3, F-actin-$S1_{a/t}$; 4, F-actin-$S1_t$; 5, F-actin-$S1_{m/t}$; 6, F-actin-S'1(75); 7, F-actin-pPDM-S1; Rel - relaxation; Con - contraction.

minished). Thus, the conformation changes of contractile proteins occurring upon isometric contraction are similar to those observed upon rigor complex formation in the F-actin - S1 system (Tables 2,3).

According to an earlier interpretation that changes of F-actin filament are reflected in decreasing Φ_E (for phalloidin-rhodamine bound to actin) and increasing $\sin^2\Theta$ (Borovikov et al. 1982 b; Galązkiewicz et al. 1987), this means that the filament goes over to the "switched on" state. The same changes occur at tension development. Hence, tension development (Table 5) and formation of complexes of F-actin with intact and trypsin-treated heads of myosin (Table 2) are associated with an increase of the respective numbers of "switched on" actin monomers, whereas domain uncoupling enhances this effect.

According to the hypothesis concerning the existence of two types of binding of myosin heads with actin filaments, the force is generated in muscle upon the transition from "weak" binding (as in myosin-ATP or myosin-ADP-P_i complexes) to "strong" binding (as in myosin-ADP and myosin nucleotide-free complexes (Mahmood and Yount 1984)). In contracting muscle the two types of binding alternate due to conformational changes of the heads upon ATP hydrolysis, and this is associated with alternating "switching on" of actin filaments.

Discussion

Our suggestion concerning the involvement of the 20 kDa domain of the myosin head in the induction of changes in the structural state of F-actin has been consistent with the evidence showing that one contact area for actin at the surface of S1 includes a peptide sited between Cys 697 and Cys 707 which corresponds to SH_1 and SH_2 (Katoh et al. 1985; Suzuki et al. 1987). It has been supposed that this region at the interface with actin is very sensitive to the presence of nucleotide triphosphate and can be considered as s "strongly" binding area.

Our data support this idea of a "strongly" binding site in the 20 kDa domain since according to our previous experience the changes in parameters of polarized fluorescence of F-actin filaments, a decrease of Φ_E and an increase of $\sin^2\Theta$, correspond to "strong" binding (Borovikov et al. 1982 b; Galązkiewicz et al. 1987).

It is accepted now that aminoacid residues of two regions of S1 are involved in the interaction with actin. They are sited at 18–22 and 37–42 kDa from the C-terminus of the S1 heavy chain respectively (Mornet et al. 1981; Chaussepied 1989). However, they have been assumed to represent but a minor determinant of the acto-S1 interface in the rigor complex (Miller et al. 1987). They function both in the absence and in the presence of nucleotide. Recently the part of actin binding site located at 18–22 kDa from C-terminus was studied with "antipeptide technology", and it was proposed that this stretch of residues 633–642 is an important determinant of the so-called "weak" acto-S1 interaction (Chaussepied 1989).

The actin binding site of the 20 kDa domain is close to the part of the active site of myosin ATPase which is located within the N-terminal domain. According to recent data, the N-terminal domain also contains a site for actin binding; this

site becomes exposed following previous separation of the N-terminal domain from the rest of S1 (Mühlrad 1989). On the contrary, in the C-terminal 20 kDa domain a stretch of aminoacids could be disclosed analogous to the sequence of the active sites of ATP-dependent enzymes (Burke et al. 1989). The 20 kDa domain, which participates in the transmission of conformational changes from S1 to actin, has also binding sites for light chains of two types: alkaline (essential) and regulatory (Mitchell et al. 1989). In our preparations of S1, the regulatory chains were absent as their preparation requires the use of EDTA (Wagner and Weeds 1977).

The alkaline light chains participate in the binding of actin; chain A1 binds more tightly than does chain A2. A site of interaction with alkaline light chains was shown to be located in the C-terminal region of actin monomer (Ueno et al. 1985). In our experiments with proteolytic degradation some splitting of A1 was observed (Fig. 1). This may have influenced the structural changes of actin. However the character of these structural changes in actin was no affected even following the removal of the 50 kDa domain. Therefore, degradation of A1 need not have influence on the structural changes in actin. This is consistent with the report of Golitsina et al. (1989) who observed isoforms S1 (A1) and S1 (A2) to induce similar structural changes of actin. Hence, the difference between A1 and A2 is not essential for the differences in conformational changes induced by these isoforms. Further investigations are needed to establish whether the alkaline chains themselves are important for the induction of conformational changes in actin. According to our previous data, the phosphorylation of regulatory (DTNB) light chains and the presence of Ca^{2+} ions are important for conformational changes to be induced by subfragment-1 and heavy meromyosin in actin filaments (Borovikov et al. 1982 b; Kąkol et al. 1987).

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