Regulation of Binding of Subfragment 1 in Isolated Rigor Myofibrils

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Abstract. A steric-hindrance model has been used to explain the regulation of muscle contraction by tropomyosin–troponin complex. The regulation of binding was studied by microscopic observation of mixtures of fluorescent subfragment 1 (S1) with rigor myofibrils at different actin-to-S1 ratios and in the presence and absence of calcium. Procedures were adapted to protect the critical thiols of S1 before conjugation to thiol-specific fluorochromes, this giving fluorescent S1 with unaltered enzyme activity. S1 binding was greatest in the I band (except at the Z-lines) in the presence of calcium regardless of the [S1]. The patterns in the absence of calcium depended on the actin-to-S1 ratios: low [S1], binding in the myosin–actin overlap region; intermediate [S1], highest binding at the A-I junction; high [S1], greatest binding in the I-band. The two distinct binding patterns observed at low [S1] were demonstrated by dual-channel fluorescence microscopy when myofibrils were sequentially incubated with fluorescent S1 without calcium followed by a different fluorescent S1 with calcium. These observations support the concept of rigor activation of actin sites. The change in the pattern upon increasing [S1] without calcium demonstrate cooperative interactions along the thin filament. However, these interactions (under the conditions used without calcium) do not appear to extend over >2–3 tropomyosin–troponin-7 actin functional units.

MUSCLE contracts by a sliding of the actin-containing thin filaments over the myosin-containing thick filaments (Huxley and Neidergerke, 1954; Huxley and Hanson, 1954), a process using the ATP driven, cyclical interaction between actin and the myosin head to produce both movement and force. The regulation of contraction in vertebrate skeletal muscle is a complex process employing a minimum of six different protein species and calcium (Ebashi et al., 1969; Weber and Murray, 1973). Both tropomyosin and troponin are required for demonstrating calcium sensitivity of ATPase activity in acto-myosin preparations. The tropomyosin–troponin complex spans seven actin monomers, and this group has been defined as the functional unit of the thin filament (Bremel and Weber, 1972). The troponin complex is composed of three subunits, troponin-T, troponin-I, and troponin-C. The latter confers calcium sensitivity to regulated acto-myosin ATPase activity, while the combination of troponin-T and troponin-I without troponin-C inhibits ATPase activity (Greaser and Gergely, 1973).

The structural mechanism by which tropomyosin and troponin regulate contraction has been studied by x-ray diffraction of intact muscle fibers. Early studies demonstrated that there was a difference in the intensity pattern of the actin layer lines between relaxed and contracted muscles; it was explained by a small movement of tropomyosin from a peripheral position on the thin filament to a position nearer to the groove of the actin filament (Haselgrove, 1972; Huxley, 1972; Parry and Squire, 1973). These structural observations and the proposed mechanism for the regulation of acto-myosin ATPase activity led to the development of the steric hindrance model for the regulation of contraction in vertebrate skeletal muscle (Haselgrove, 1972; Huxley, 1972; Parry and Squire, 1973). In its simplest form the model predicts that, in the absence of calcium, tropomyosin occupies a position on the thin filament that interferes with the interaction between actin and the myosin head thus preventing contraction. Calcium binding to troponin-C causes a movement of tropomyosin to a position that no longer interferes with the interaction between actin and the myosin head. Other structural studies of regulated thin filaments (actin-tropomyosin-troponin subunits) showed a change in the position of tropomyosin when calcium was present (Gillis and O'Brien, 1975). Also, the presence of troponin-T and I changed the position of tropomyosin on the actin filament (Wakabayashi et al., 1975). The steric hindrance model received further support in the time-resolved x-ray diffraction studies of Kress et al. (1986), who showed that tropomyosin movement in the muscle fiber precedes tension generation.

One interpretation of the model is that tropomyosin physically blocks the binding of the myosin head to actin. This has been tested both by ATPase activity measurements and by binding studies using soluble fragments of the myosin head (subfragment 1, S1)[1] and regulated thin filaments. The results

1. *Abbreviations used in this paper*: A/fS1, actin monomer/fluorescent myosin subfragment 1; F-5-M, fluorescein-5-maleimide; IRB, imidazole rigor buffer; PRB, phosphate rigor buffer; R-X-M, rhodamine-X-maleimide; S1, myosin subfragment 1; SL, sarcomere length.

indicate that a simple blocking of the binding of the myosin head to actin cannot be used to explain calcium regulation in reconstituted actin–S1 systems. Bremel and Weber (1972) and Bremel et al. (1972) demonstrated that the inhibition of actin-activated S1 ATPase activity afforded by the presence of tropomyosin and troponin on the actin filament could be removed by either calcium addition or low ATP concentration. In the absence of calcium, the ATPase activity was biphasic with respect to ATP concentration: it increased with ATP concentration, reached a peak value, and then declined at high ATP levels. In the presence of calcium, the ATPase activity was related hyperbolically to ATP concentration. The activity in the absence of calcium was explained by the presence of rigor bonds (actin–S1 complex without bound nucleotide) on the regulated thin filament that activated additional actin sites for nucleotide bound S1 to interact with. A biphasic dependence on ATP concentration has been observed in the absence of calcium for both ATPase activity in myofibrils (Weber, 1970) and tension generation in skinned muscle fibers (Godt, 1974; Moss and Haworth, 1984; Reuben et al., 1971; White, 1970), demonstrating that a more physiological arrangement of actin relative to the myosin head results in similar phenomena. Kinetic studies by Chalovich et al. (1981) and Chalovich and Eisenberg (1982) showed that, during steady-stage ATPase activity, the affinity of S1 for actin in regulated thin filaments was independent of calcium whereas the ATPase activity was highly dependent upon calcium. This led them to suggest that regulation of contraction occurs by regulating a kinetic step in the ATPase cycle of myosin. Rosenfeld and Taylor (1987*a*,*b*) also studied the regulation of actin-activated ATPase activity by troponin–tropomyosin and concluded that regulation may be explained by a partial steric-blocking mechanism or by a conformational mechanism not dependent upon steric-blocking. Other kinetic (Trybus and Taylor, 1980) and equilibrium binding studies demonstrated that S1 without ATP and calcium (Greene, 1982) or with bound ADP (Greene and Eisenberg, 1980) binds to the regulated thin filament in an apparently cooperative manner: initial binding or low saturation of the actin sites favors more extensive binding of S1 to regulated thin filaments. The cooperative nature of S1 binding (i.e., the unblocking of additional sites along the thin filament by the presence of rigor complexes) has been explained by assuming that the tropomyosin strand is relatively rigid. Upon formation of a rigor complex, the strand is displaced into the groove that unblocks additional actin sites (Bremel and Weber, 1972; Bremel et al., 1972; Murray et al., 1980; Murray et al., 1981).

Hill et al. (1980, 1983) have developed mathematical models to describe the highly cooperative nature of S1 binding to regulated thin filaments. The model incorporates positive cooperativity within and between functional units. The most recent formalism (Hill et al., 1983) includes three parameters: the binding constant for S1 to an unblocked actin site, the binding energy expended by the first S1 molecule that binds the blocked functional unit, and the nearest neighbor tropomyosin–tropomyosin interaction energy between functional units. This mathematical model was shown to describe the data of Greene and Eisenberg (1980) for the equilibrium binding of S1-ADP to regulated thin filaments (Hill et al., 1983). The structural implication of the data and the model is that, at low levels of saturation of the actin sites on the regulated thin filament, S1 should not be randomly distributed along the filament but should be localized to regions in which the initial binding occurred.

Structural studies using x-ray diffraction showed that tropomyosin is displaced from the relaxed position in rigor muscle in the absence of calcium, and the amount of displacement is dependent upon the degree of overlap between thick and thin filaments, being greatest at maximum overlap (Haselgrove, 1972). Güth and Potter (1987) have used a fluorescent conjugate of troponin-C as a means of measuring thin filament unblocking in skinned fibers. Their studies showed that the troponin-C fluorescence in the fiber increased both when calcium was added and when the fibers were bathed in a solution without ATP or calcium; the latter increase was sarcomere length dependent and greatest at maximum myosin head–thin filament overlap. These studies suggest that tropomyosin can be displaced in a regional fashion in the absence of calcium, due to the formation of rigor crossbridges in the fiber.

To test for the regulation of S1 binding and the concept of cooperative binding of S1 to regulated thin filaments, a study was made of the location of fluorescent-S1 binding (in the absence of nucleotide) in myofibrils both with and without calcium. The S1 was conjugated to fluorophores at thiols other than SH_1 or SH_2 such that the ATPase activity was not altered. Bovine rectus abdominis myofibrils were used taking advantage of their longer thin filaments (Bendall and Voyle, 1967), and bovine cutaneous trunci was used to prepare S1 since myosin from this muscle is essentially devoid of light chain 3 (Young and Davey, 1981). These studies demonstrate both regulation of S1 binding to actin sites and cooperative (nonrandom) binding of S1 to actin sites in the myofibril.

Materials and Methods

Unless specified otherwise, all procedures were carried out at 0–4°C.

Myofibril Purification

Methods for myofibril purification were modified from Wang and Greaser (1985) to ensure that the myofibrils were in rigor before homogenization to prevent filament disorientation (Locker et al., 1976). Strips of muscle from rabbit psoas or bovine rectus abdominis were excised 5–30 min postmortem. The strips were dissected along the fiber axis into large fiber bundles of ~0.5 × 5 cm that were either stretched or allowed to slacken to various extents, and were then tied to applicator sticks with dental tape. The affixed fiber bundles were bathed in phosphate rigor buffer (PRB, 75 mM KCl, 5 mM KH_2PO_4 [pH 7.2], 2 mM EGTA, 2 mM $MgCl_2$, and 2 mM NaN_3) plus 0.5% Triton X-100 (vol/vol) and 0.1 mM PMSF for 18–24 h with stirring. The muscle bundles were macerated with scissors, and then were briefly homogenized with a Polytron (Brinkman Instruments Co., Westbury, NY) for four 1–2 s bursts in 15 vol of PRB. Subsequent disintegration of the muscle was done using a Dounce homogenizer; the mince was diluted with approximately five more vol of PRB and was then homogenized using 50–100 strokes with a loose fitting pestle. Myofibrils and fiber pieces were pelleted by centrifugation at 1,000 *g* for 10 min, and the pellet was resuspended in ~20 vol of PRB and homogenized for another 50–100 strokes. The suspension was then diluted to ~30 vol and filtered through a layer of cheesecloth. The myofibrils were collected by centrifugation at 1,000 *g* for 10 min, resuspended in 20 vol PRB made to 0.5% Triton X-100, pelleted by centrifugation at 1,000 *g* for 5 min, resuspended in PRB, and washed three more times with rigor buffer using 15 vol per wash. The final myofibril pellet was suspended in PRB made to 50% glycerol (vol/vol) and 1 mM DTT to a final protein concentration of 1–5 mg protein/ml. The suspension was stored at −20°C in a nondefrosting freezer.

Protein Purification

Myosin was purified from bovine and rabbit muscle using the methods adapted from Kielley and Bradley (1956) and Mihalyi and Rowe (1966). S1

was generated from myosin using the procedures of Weeds and Pope (1977) and Okamoto and Sekine (1985). Actin was purified following the methods of Pardee and Spudich (1982) from the myosin-extracted muscle mince.

S1 Modification

Oxidation of the thiols with 5,5'-dithiobis(2-nitrobenzoic acid) was accomplished essentially as described by Wells and Yount (1980) except that ATP was substituted for ADP. After thiol modification, the 5-thio-2-nitrobenzoic acid content was determined spectrophotometrically (Ellman, 1959). The oxidized S1 was dialyzed against PRB to remove the 5-thio-2-nitrobenzoic acid. The proteins (4–8 mg/ml), both thiol protected and an unprotected control, were then mixed separately with an equal volume of PRB containing either fluorescein-5-maleimide (F-5-M) or rhodamine-X-maleimide (R-X-M), previously suspended in acetonitrile at 10–20 mg/ml at a ratio of ~2.5 mol dye to 1 mol S1. The mixture was incubated for 2 h in the dark, and the reaction was quenched by adding DTT to 1 mM followed by dialysis against PRB plus 1 mM DTT. To further purify the conjugated S1, an actin affinity precipitation step was employed. Filamentous actin was suspended in PRB plus 1 mM DTT and mixed with the conjugated S1 in a 3:1 mol ratio (actin monomer to S1). This suspension was centrifuged at 150,000 *g* for 1 h and the supernate was discarded. The pellet was resuspended in an equal volume of PRB made to 150 mM KCl, 10 mM $Na_4P_2O_7$ (pH 7.2), 1 mM DTT, using a Dounce homogenizer. The suspension was centrifuged at 150,000 *g* for 1 h, and the supernate was collected and adjusted to 40% saturation using solid ammonium sulfate to remove the actin. The precipitate was removed by centrifugation at 10,000 *g* for 20 min, and the supernate was adjusted to 75% saturation of ammonium sulfate. The precipitate was collected by centrifugation and stored as a suspension on ice in the dark.

ATPase Activity

The different S1 conjugates (control, R-X-M, and F-5-M) were dialyzed against 10 mM imidazole (pH 7.0) 1 mM DTT before measurement of the enzyme activity. The control S1 was not treated with 5,5'-dithiobis(2-nitrobenzoic acid) or further purified by the actin affinity step. The calcium, potassium-EDTA, and actin-activated magnesium-ATPase activities were determined in solutions as described by Margossian and Lowey (1978) in a final volume of 1 ml at 25°C. The concentration of S1 used in the assays ranged from 12.5 to 50 μg/ml and the range of actin concentrations used for the double reciprocal plots was 4–30 μM. Inorganic phosphate production was measured at three to four time points after initiation of the reaction by the malachite green assay of Carter and Karl (1982). Standard curves were prepared using dried K_2HPO_4.

Single Label Experiments with and without Calcium

Myofibril preparations of different sarcomere lengths (SL) were mixed, and were then washed with imidazole rigor buffer (IRB) made to 1 mg BSA/ml and with (+) or without (−) calcium (IRB, 75 mM KCl, 10 mM imidazole [pH 7.2], 0.2 mM $CaCl_2$ (IRB+) or 2 mM EGTA (IRB-), 2 mM $MgCl_2$, 2 mM NaN_3, 1 mM DTT). The glycerinated myofibrils were washed by repeated resuspension and centrifugation at 1,000 *g* for 5 min, using three washes with 5 vol buffer/wash. The final suspension was diluted to 1 mg myofibrillar protein/ml. Fluorescent S1 was dialyzed overnight at 4°C in the dark against either IRB+ or IRB−. Myofibrils (100 μl) were rapidly mixed with 300 μl of IRB+ or IRB− made to 1 mg BSA/ml and containing different amounts of R-X-M S1, the mixture being contained in a 500 μl microfuge tube. The suspension was incubated at 20–24°C in the dark for 20 min and the myofibrils were pelleted by centrifugation at 13,500 *g* for 8–10 s (model "E" microfuge; Beckman Instruments, Inc., Palo Alto, CA). The supernate was removed, the myofibrils were resuspended in the appropriate IRB (+ or − calcium), and 100 μl was spread on a coverslip (No. 1.5). Myofibrils were fixed with 3% formaldehyde in the appropriate IRB by pooling 400 μl of fixative on the coverslip. The time interval between resuspension and fixation was ~30 s, this minimizing the extent of reequilibration of fluorescent S1. After 10 min, the fixative was drained off, and the coverslip was rinsed in IRB and placed on 50 μl of mounting medium (70% [vol/vol]) glycerol, 75 mM KCl, 10 mM Tris (pH 8.5), 2 mM EGTA, 2 mM $MgCl_2$, 2 mM NaN_3, 1–2 mg *p*-phenylenediamine/ml) on a glass slide. Excess mounting medium was pressed out and the coverslip was sealed with "Wet and Wild" nail polish (No. 401; Pavion Ltd., NY, NY).

Several concentrations of fluorescent S1 were used to stain myofibrils, all being subsaturating with respect to total myofibrillar actin. The content of actin was calculated from the data of Yates and Greaser (1983) as 0.54 nmol (1.35 μM) for the mass of myofibrils used (100 μg in a final volume of 400 μl). The S1 was added at mole ratios of myofibrillar actin/fluorescent S1 (A/fS1) of 24, 16, 8, 4, and 2:1 corresponding to 56, 84, 168, 336, and 672 nM S1, respectively.

Double Label Experiments with and without Calcium

Myofibrils and S1 were prepared as described above. For staining the unblocked actin sites in the myofibril, the myofibrils were first mixed with R-X-M S1 at a mole ratio of 24 A:1 fS1 (56 nM) in the absence of calcium. The suspension was incubated for 20 min at 20–24°C, and the myofibrils were pelleted by centrifugation at 13,500 *g* for 8–10 s. The supernate was removed, and the pellet was resuspended in 400 μl of IRB+ containing 1 mg BSA/ml and F-5-M S1 at a mole ratio of 24 A:1 fS1 (56 nM). Aliquots (100 μl) were removed at 0, 8, and 16 min after resuspension in IRB+, and were fixed and mounted as described in the preceding paragraph.

Observation and Photography

Stained myofibrils were observed and photographed (using a 100× objective) with a standard Zeiss microscope equipped with fluorescein and rhodamine fluorescence filters and phase-contrast optics. Images were recorded with Kodak Technical Pan Film 2415, which was developed with HC110 (dilution B) for 6–8 min at 20°C.

SDS-PAGE

Chromatographic elution profiles and protein purity were routinely monitored with SDS-PAGE using 10% acrylamide gels (pH 9.3; 10% T, 0.5% C resolving gel). Modifications to the standard Laemmli (1970) procedures are described in Giulian et al. (1983), Yates and Greaser (1983), and Fritz et al. (1989). Proteins conjugated with fluorophores were electrophoresed, briefly rinsed with distilled water, and placed directly on a UV lamp. The fluorescent bands were photographed through a combination of a UV filter and an orange Wratten filter using Kodak Technical Pan film 4415. The film was developed with either Kodak D-19 or HC-110 (dilution B). Gels were subsequently stained with Coomassie brilliant blue (R-250), destained, and photographed again. Before photography, the stained gels were shrunk with 12% (vol/vol) polyethylene glycol to increase sensitivity (Palumbo and Tecce, 1983).

Miscellaneous

Myofibrillar protein concentration was determined by the biuret assay (Gornall et al., 1949) using BSA as the standard. The protein concentrations of actin, chymotrypsin, BSA, myosin, and S1 were determined spectrophotometrically at 280 nm (with light-scattering correction at 320 nm), using mass absorptivity values 1.17, 2.12, 0.67, 0.56, and 0.75 ml/mg-cm, respectively. Molar concentrations of actin and S1 were estimated using molecular weights of 43,000 and 120,000. The concentration of conjugated S1 was determined by the microtannin–turbidity assay of Mejbaum-Katzenellenbogen and Dobryszycka (1959) as modified by Trayer and Trayer (1988), using unconjugated S1 as the standard. The mole ratio of covalently attached dye to protein was determined by absorbance at 490 nm for fluorescein conjugates and 580–590 nm for rhodamine conjugates, using molar absorptivities (1 cm) of 60,000 and 70,000 for fluorescein and rhodamine, respectively.

Biochemicals and Reagents

F-5-M and R-X-M were purchased from Molecular Probes Inc. (Eugene, OR). Alpha-chymotrypsin (type II), ATP, BSA, Coomassie brilliant blue (R-250), *N,N'*-diallytartardiamide, 5,5'-dithiobis(2-nitrobenzoic acid), DTT, EDTA, EGTA, imidazole, glycine, polyethylene glycol, *p*-phenylenediamine, SDS, thiourea, and Tris were supplied by Sigma Chemical Co. (St. Louis, MO). Ammonium sulfate (Ultra Pure) was purchased from ICN Biomedicals Inc. (Cleveland, OH). Acrylamide and *N,N'*-methylenebisacrylamide were from Bethesda Research Laboratories (Gaithersburg, MD). Poly(vinyl alcohol), malachite green HCl and *N,N,N',N'*-tetramethylethylenediamine were from Aldrich Chemical Co. (Milwaukee, WI) and formaldehyde (E. M. grade) was from Ladd Research Industries, Inc. (Burlington, VT).

Results

Synthesis of Fluorescent S1

The documented high reactivity of the critical thiols of S1,

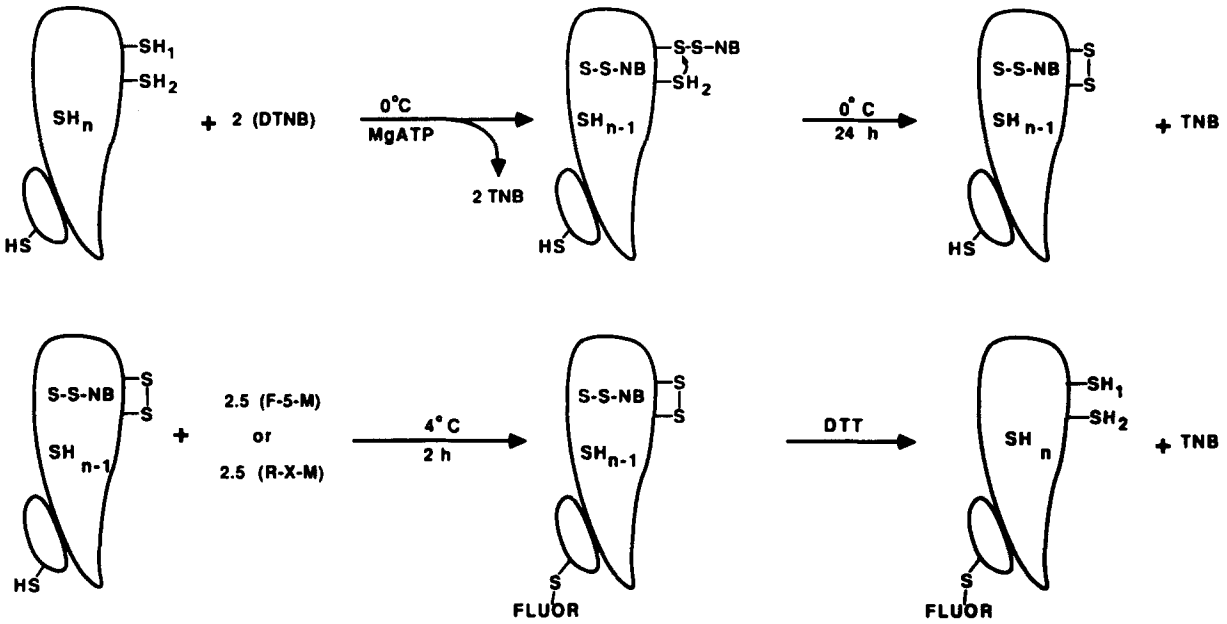


Figure 1. Schematic diagram for the modification of S1 at thiols other than SH_1 or SH_2. S1 heavy chain is represented by the pear-shaped molecule and light-chain 1 is represented by the small oval. The critical thiols (*SH_1* and *SH_2*) are first oxidized by reacting with 2 mol 5,5′-dithiobis(2-nitrobenzoic acid) (*DTNB*)/mole S1 in the presence of ATP, the reaction occurring by the initial formation of a mixed disulfide followed by formation of an intramolecular disulfide between SH_1 and SH_2 with a total of 3 mol of 5-thio-2-nitrobenzoic acid (*TNB*) produced (Wells and Yount, 1980) as depicted in the upper panel. The remaining protein sulfhydryls are then reacted with either F-5-M or R-X-M using 2.5 mol of fluorophore/mole S1 followed by reduction of protein disulfides and mixed disulfides with DTT as shown in the lower panel. The final product had most of the fluorophore in the light chain and retained its catalytic activity as discussed in the text.

SH_1, and SH_2 (Takamori et al., 1976), and the changes in ATPase activity that occur upon modification of these thiols (Kielley and Bradley, 1956; Sekine and Kielley, 1964; Silverman et al., 1972), led to the development of a procedure for conjugation of fluorophores to thiols other than SH_1 or SH_2. Fig. 1 shows a schematic diagram of the procedure for the protection of SH_1 and SH_2 via 5,5′-dithiobis(2-nitrobenzoic acid)–catalyzed oxidation (Wells and Yount, 1980; Huston et al., 1988) followed by conjugation with sulfhydryl-specific fluorophores and subsequent deprotection of the critical thiols by reduction with DTT. The conjugated protein was further purified by taking advantage of the weak binding of S1 to actin in the presence of pyrophosphate. This procedure was used to select for conjugated S1 that bound actin reversibly, since exhaustive modification of S1 with *N*-ethylmaleimide gives S1 which binds actin irreversibly (Meeusen and Cande, 1979; Pemrick and Weber, 1976). Analysis of the supernatant and pellet after pyrophosphate treatment showed that ~90% of the S1 was present in the supernate with some contaminating actin, which was removed by ammonium-sulfate fractionation (data not shown). The conjugated protein was analyzed by SDS-PAGE followed by UV illumination to determine whether the thiol protection scheme had resulted in the fluorophore being conjugated to the heavy chain of S1 (where SH_1 and SH_2 are located) or to light-chain 1. Fig. 2 shows the protein stain and fluorescent gel patterns observed from S1 that was conjugated with (Fig. 2, lanes *a* and *b*) or without (Fig. 2, lanes *c* and *d*) thiol protection and with either R-X-M (Fig. 2, lanes *a* and *c*) or F-5-M (Fig. 2, lanes *b* and *d*). The fluorescence pattern observed in thiol-protected S1 was quite

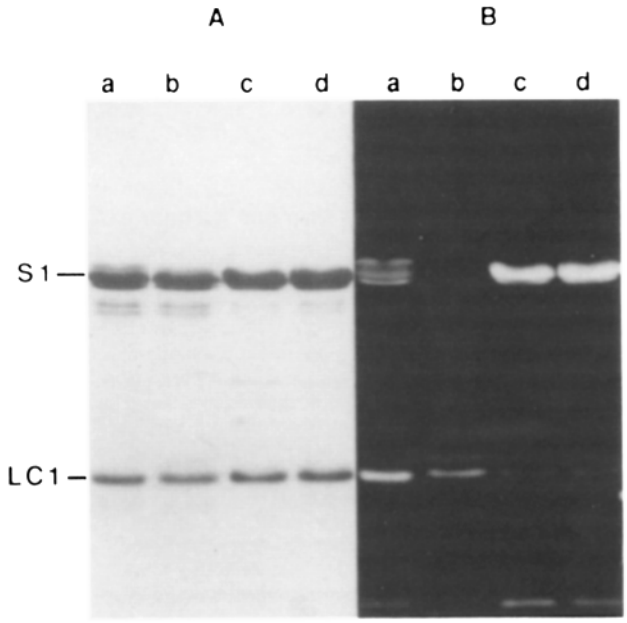


Figure 2. Demonstration of the change in the location of fluorescence in the subunits of S1 induced by thiol protection. S1 was either thiol protected (lanes *a* and *b*) or not protected (lanes *c* and *d*) and then reacted with either R-X-M (lanes *a* and *c*) or F-5-M (lanes *b* and *d*) then analyzed by SDS-PAGE as described in Materials and Methods. The gel was photographed under UV-illumination (*B*) before staining with Coomassie brilliant blue (*A*). Note that in the lanes containing the thiol-protected S1 most of the fluorescence was in the light-chain band (*LC1*) while in the nonprotected S1 most of the fluorescence was in the heavy-chain subunit of S1 (*S1*).

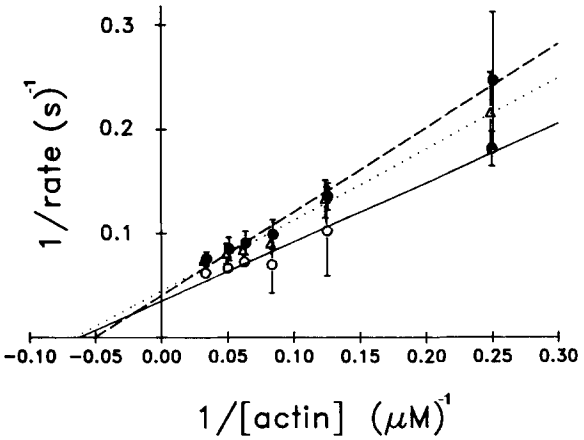


Figure 3. Analysis of the actin-activated ATPase activity of fluorescent conjugates of S1 by double reciprocal plots. The ATPase activity of S1 conjugates was measured as described in Materials and Methods using different actin concentration (4–30 μM), and the data were plotted as double reciprocal plots. The data points represents the pooled mean of two separate preparations assayed in triplicate (with SD error bars). The maximum velocity and K_m were estimated from the intercepts of the best fit line as determined by linear regression and are shown in Table I. Note that the F-5-M (*solid circle*) and R-X-M (*open triangle*) had rates similar to the control S1 (*open circle*) at all actin concentrations tested.

different from that observed in the unprotected S1. Most of the fluorescence was in the light chain 1 in the protected S1, while most was in the heavy chain of the unprotected S1. The estimated dye-to-protein ratio for the protected S1 was 0.4–0.6 for F-5-M conjugates and 0.3–0.4 for R-X-M conjugates (n = 2). The fluorescent gel patterns depict the R-X-M conjugate as containing more fluorescence; this is the result of the higher quantum yield of R-X-M and the filters used to photograph the fluorescent gel. Both protected and unprotected S1 were dissociated from actin by pyrophosphate treatment, suggesting that conjugation of thiols under the conditions used did not cause appreciable irreversible binding of modified S1 to actin.

The kinetic characteristics of the fluorescent conjugates of S1 were investigated to verify that the protection protocol did not alter the active site of the enzyme. Fig. 3 shows double reciprocal plots of the unregulated actin-activated Mg^{2+}-ATPase activity of control, F-5-M and R-X-M S1 species. Conjugation did not change the characteristics of the ATPase activity upon actin activation. The Ca^{2+} and K^+-EDTA ATPase activities and the V_m and K_m for actin activation are shown in Table I. Conjugation did not alter any of these activities or characteristics; the critical thiols were thus not modified, since modification of SH_1 or SH_1 and SH_2 dramatically alters the enzymes' activities measured under similar conditions (Kielley and Bradley, 1956; Sekine and Kielley, 1964; Silverman et al., 1972; Wells and Yount, 1980). The yield of fluorescent S1 relative to the amount of S1 before thiol modification was 30–50%. The enzyme activity was stable for at least 4 mo, while the staining (see below) was stable for over 8 mo when the S1 was stored as an ammonium sulfate pellet at 0°C in the dark.

The data demonstrate that the conjugation procedures protect the critical thiols and thus the enzyme activity, and in addition give a reasonable dye-to-protein ratio and final yield of conjugate. This is highly relevant to the recent observation (Titus et al., 1989) that modification of myosin at SH_1 can result in complete loss of the calcium sensitivity of the regulated (tropomyosin/troponin) actin-activated ATPase activity of myosin. The calcium sensitivity of regulated actin-activated ATPase activity of the S1 conjugates was not measured in this study; however, the calcium sensitivity of the location of binding of the S1 conjugates was measured and is described below.

Binding Pattern of Fluorescent S1 on Myofibrils

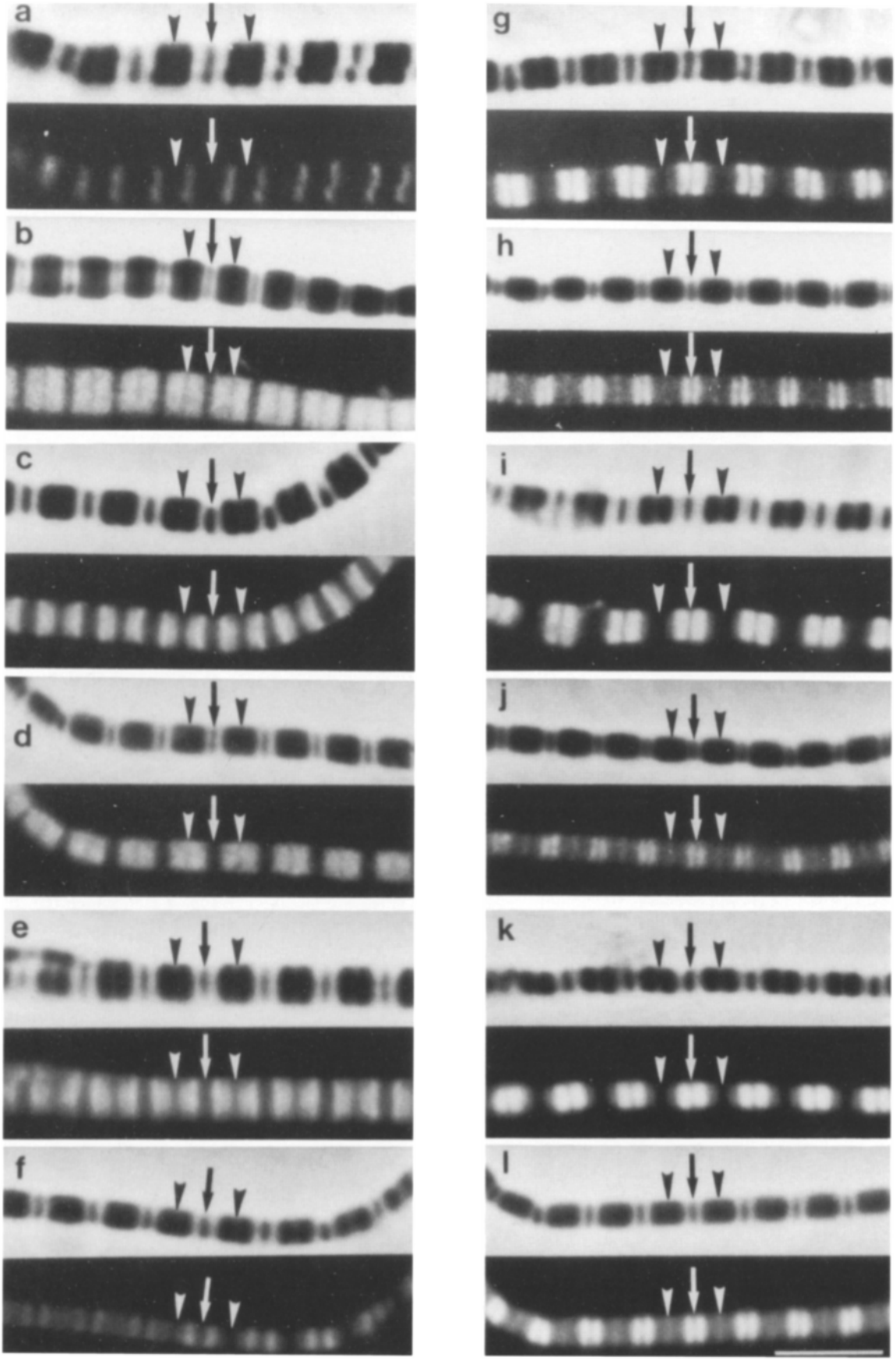
The ability of tropomyosin/troponin to block actin sites in the myofibril was investigated using fluorescent S1, in the absence of nucleotide, as a probe for unblocked actin sites. The locations of S1 binding to myofibrils of different sarcomere length (SL) and as a function of A/fS1 and in the absence and presence of calcium are shown in Figs. 4 and 5. The prints for the fluorescent images were made to demonstrate the differences in light intensity and thus in S1 binding. In the absence of calcium and at 24 A:1 fS1, the fluorescent protein was found to be distributed as a pair of bands in each sarcomere (Fig. 4, *a* and *b*). The width of each band in the half sarcomere was dependent upon SL, being wider in short SL myofibrils and corresponded to the degree of myofibrillar actin–myosin head overlap. When myofibrils were incubated with S1 in the absence of calcium and at 16 A:1 fS1 (Fig. 4, *c* and *d*), a pair of fluorescent bands was observed in each sarcomere, yet the intensity of the fluorescence in each band was not homogeneous for longer SL myofibrils (Fig. 4 *c*): a more intense staining region was seen at the edge of the A-band adjacent to the I-band. This nonhomogeneous pattern was not observed in sarcomeres of shorter length (Fig. 4 *d*). Incubation in the absence of calcium and at 8 A:1 fS1 showed a pair of fluorescent bands in each sarcomere, the intensity in each band being greatest at the A-band–I-band junction, less in the overlap region, and least in the I-band near the Z-line (Fig. 4, *e* and *f*). SL was found to influence the intensity pattern of the S1 binding as described previously; this is readily apparent in Fig. 4 *f*, in which the SL increases from left to right. At shorter SL, the intensity of S1 binding at the A-I junction is less, while at longer SL, it is greater.

When myofibrils were incubated in the presence of calcium and with different A/fS1 (Fig. 4, *g–l*), the pattern of S1 binding was essentially independent of A/fS1 and was quite different from that seen in the absence of calcium. In

*Table I. Kinetic Parameters of Control and Fluorescent Conjugates of Bovine Cutaneous Trunci S1**

S1	Ca^{2+}-ATPase (*mol P_i/s*)	K^+-EDTA-ATPase (*mol P_i/s*)	Actin-activated Mg^{2+}-ATPase (*mol P_i/s*)	
			V_m	K_m(μM)
Control	1.75(0.15)‡	6.28(1.8)	28	16
R-X-M	1.70(0.29)	5.69(1.2)	23	19
F-5-M	1.73(0.23)	5.31(1.2)	24	15

* Pooled data of two preparations assayed in triplicate.
‡ Numbers in parentheses show the standard deviation of the mean.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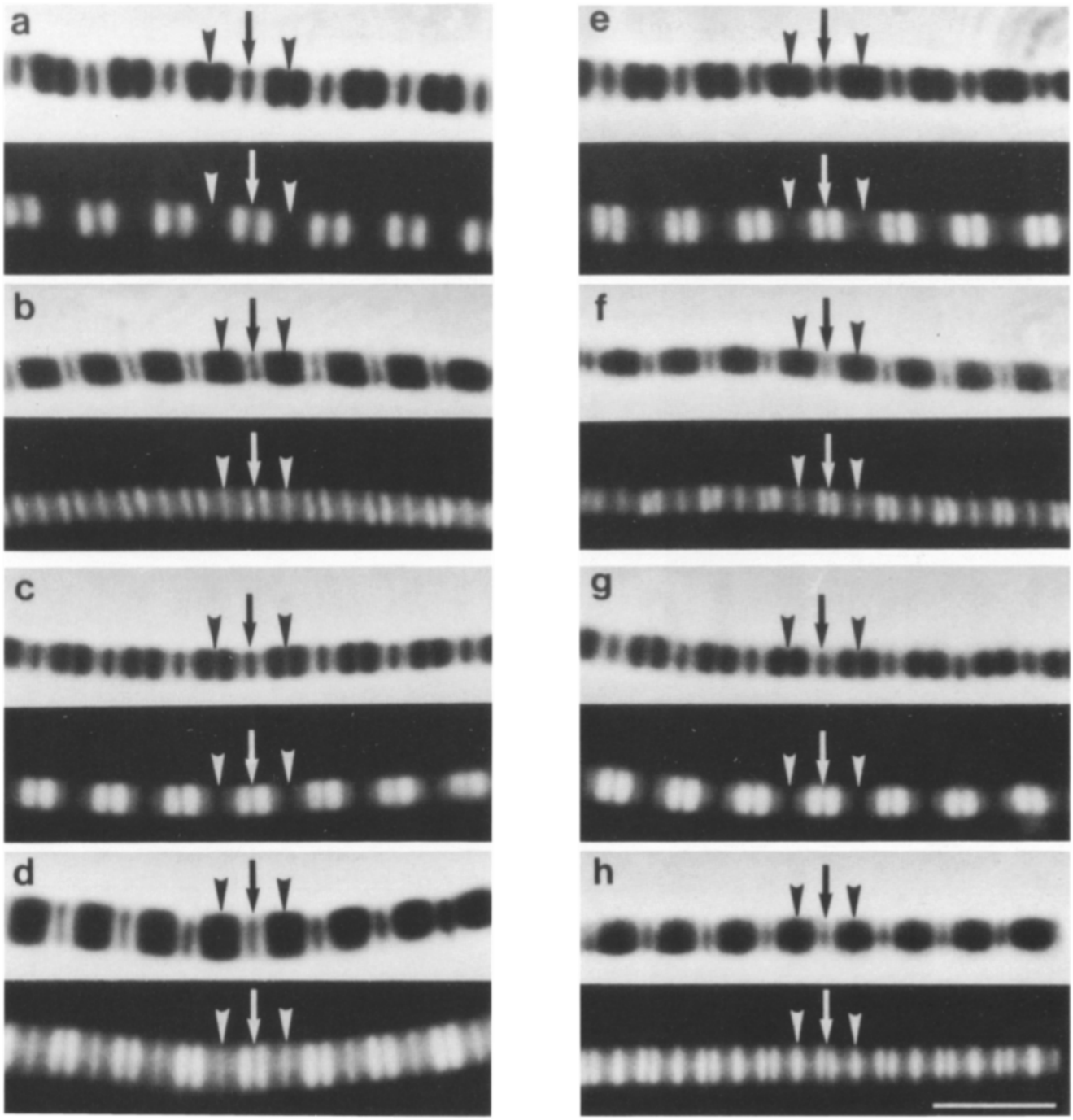

Figure 5. Pattern of binding of fluorescent S1 to myofibrils at low actin/S1 in the absence and presence of calcium. Myofibrils were incubated with R-X-M S1 at 4 A:1 fS1 (*a*, *b*, *e*, and *f*); 2 A:1 fS1 (*c*, *d*, *g*, and *h*); in either the absence (*a–d*) or presence (*e–h*) of calcium. After a 20-min incubation, the myofibrils were sedimented, the supernatant was removed, and the myofibrils were resuspended in buffer and fixed as described in Materials and Methods. Each myofibril was photographed using phase-contrast (*upper*) and fluorescence (*lower*) illumination and, at each level of A/fS1, a long SL (*upper pair*) and short SL (*lower pair*) myofibril were photographed. The arrows mark the Z-line and the arrowheads mark the center of the A-band. Note the slight change in the pattern of binding as the A/fS1 was decreased (*a* and *c*) and that the influence of calcium (*a* and *e*) on the binding pattern was much less obvious. Bar, 5 μm.

Figure 4. Pattern of binding of fluorescent S1 to myofibrils at high to intermediate A/fS1 in the absence and presence of calcium. Myofibrils were incubated with R-X-M S1 at 24 A:1 fS1 (*a*, *b*, *g*, and *h*); 16 A:1 fS1 (*c*, *d*, *i*, and *j*); and 8 A:1 fS1 (*e*, *f*, *k*, and *l*) in either the absence (*a–f*) or presence (*g–l*) of calcium. After a 20-min incubation, the myofibrils were sedimented, the supernatant removed and the myofibrils resuspended in buffer and fixed as described in Materials and Methods. Each myofibril was photographed using phase-contrast (*upper level*) and fluorescence (*lower level*) illumination, and, at each level of A/fS1, a long SL (*upper pair*) and short SL (*lower pair*) myofibril was photographed. The arrows mark the Z-line and the arrowheads mark the center of the A-band. Note the change in the pattern of binding as the A/fS1 was decreased (*a* and *f*) and the influence of calcium (*a* and *g*) on the binding pattern. Bar, 5 μm.

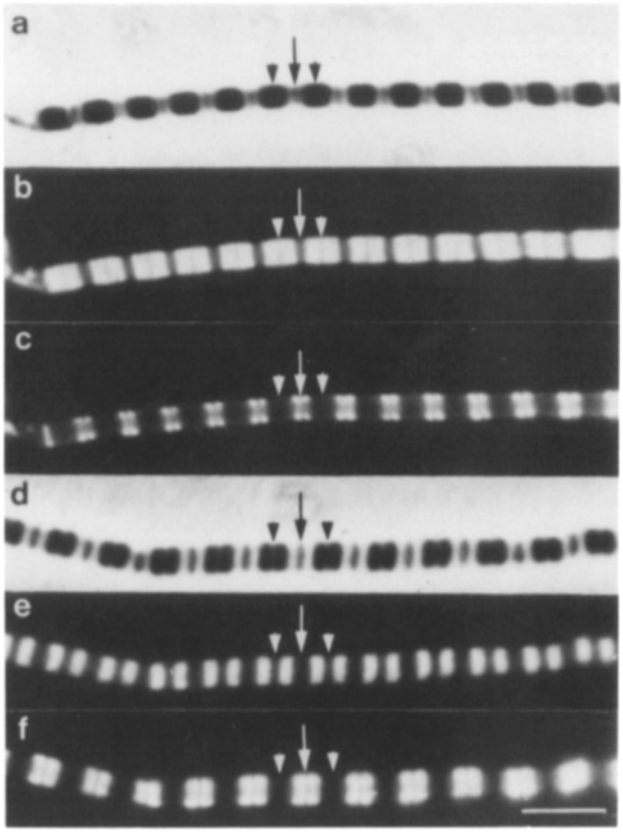

Figure 6. Pattern of binding of fluorescent S1 on myofibrils sequentially incubated with R-X-M S1 in the absence of calcium and briefly with F-5-M S1 in the presence of calcium at high A/fS1. Myofibrils were first incubated with R-X-M S1 at 24 A:1 fS1 in the absence of calcium. After 20 min, the myofibrils were sedimented, the supernatant was removed, and the myofibrils were resuspended in F-5-M S1 at 24 A:1 fS1 in the presence of calcium. Immediately after resuspension, an aliquot was removed and fixed. The myofibrils were photographed using phase-contrast (*a* and *d*), rhodamine fluorescence (*b* and *e*) and fluorescein fluorescence (*c* and *f*) illumination. A short SL (*a–c*) and long SL (*d–e*) myofibril is shown. The arrows mark the Z-line and the arrowheads mark the center of the A-band. Note that the distinct difference in the pattern of fluorescent-S1 binding was dependent on calcium when incubation time in the presence of calcium was minimized. Bar, 5 μm.

longer SL myofibrils (Fig. 4, *g*, *i*, and *k*), the pattern of S1 binding in the sarcomere was seen as a pair of intense-staining bands separated by a pair of less intense staining bands. The S1 bound actin sites throughout the myofibril except at the Z-line and H-zone (if present); most was bound in the I-band and some in the overlap region. Note that in the shorter SL myofibrils (Fig. 4, *h*, *j*, and *l*) there was a more intense staining band in the center of the A-band. This was presumably due to thin filament penetration into the bare zone (Huxley, 1964), a region in the A-band that is devoid of myosin heads, resulting in more actin sites in this region than in the flanking regions.

When myofibrils were incubated at 4 A:1 fS1 and 2 A:1 fS1 (Fig. 5, *a–h*), differences between the binding patterns observed in the absence and presence of calcium were less noticeable. Incubation in the absence of calcium and at 4 A:1 fS1 resulted in S1 binding actin essentially throughout the myofibril except at the Z-line and H-zone. However, at a longer SL, the most intense binding was seen in the I-band, adjacent to the A-band; the intensity was less in the A-band where the thin and thick filaments overlap, and less in the region of the I-band nearest to the Z-line (Fig. 5 *a*). At shorter SL (Fig. 5 *b*), this heterogeneous staining was less obvious, but the nonstaining Z-line region appeared wider than that seen in a similar myofibril (same A/fS1 and SL) in the presence of calcium (Figure 5 *f*). Staining myofibrils at 2 A:1 fS1 gave patterns that were independent of the absence or presence of calcium (compare Fig. 5, *c* and *d*, to Fig. 5, *g* and *h*). At this S1 level and in the absence of calcium, the S1 bound actin sites in the I-band (except at the Z-line) and in the overlap region; the intensity of the S1-binding appeared homogeneous in the I-band and the width of the non-

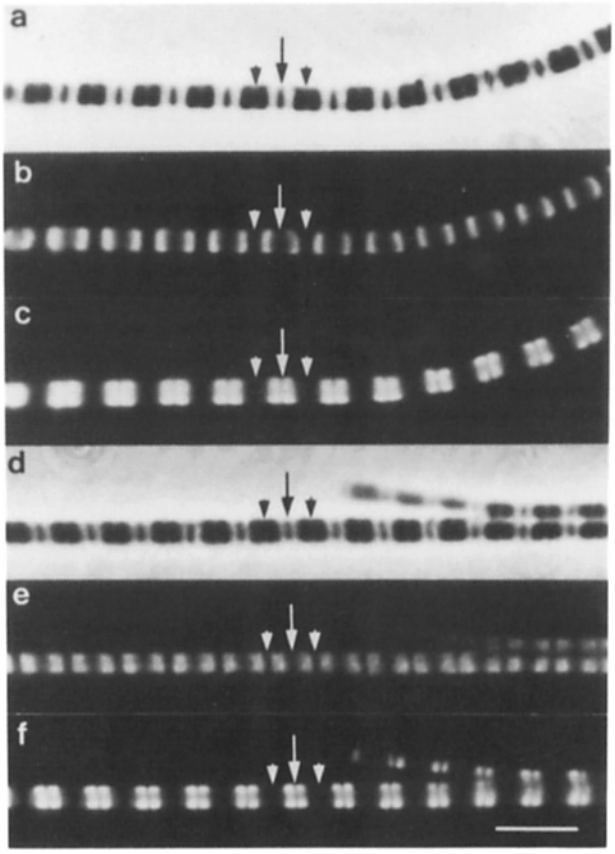

Figure 7. Pattern of binding of fluorescent S1 on myofibrils sequentially incubated with R-X-M S1 in the absence of calcium and with F-5-M S1 in the presence of calcium at high A/fS1. Myofibrils were first incubated with R-X-M S1 at 24 A:1 fS1 in the absence of calcium. After 20 min, the myofibrils were sedimented, the supernatant was removed, and the myofibrils were resuspended in F-5-M S1 at 24 A:1 fS1 in the presence of calcium. At 8 min (*a–c*) and 16 min (*d–e*) after resuspension, an aliquot was removed and fixed. The myofibrils were photographed using phase-contrast (*a* and *d*), rhodamine fluorescence (*b* and *e*) and fluorescein fluorescence (*c* and *f*) illumination. The arrows mark the Z-line and the arrowheads mark the center of the A-band. Note that a longer duration of incubation in the presence of calcium results in a less distinct difference in the pattern of fluorescent-S1 binding as influenced by calcium (compare also with Fig. 6). Bar, 5 μm.

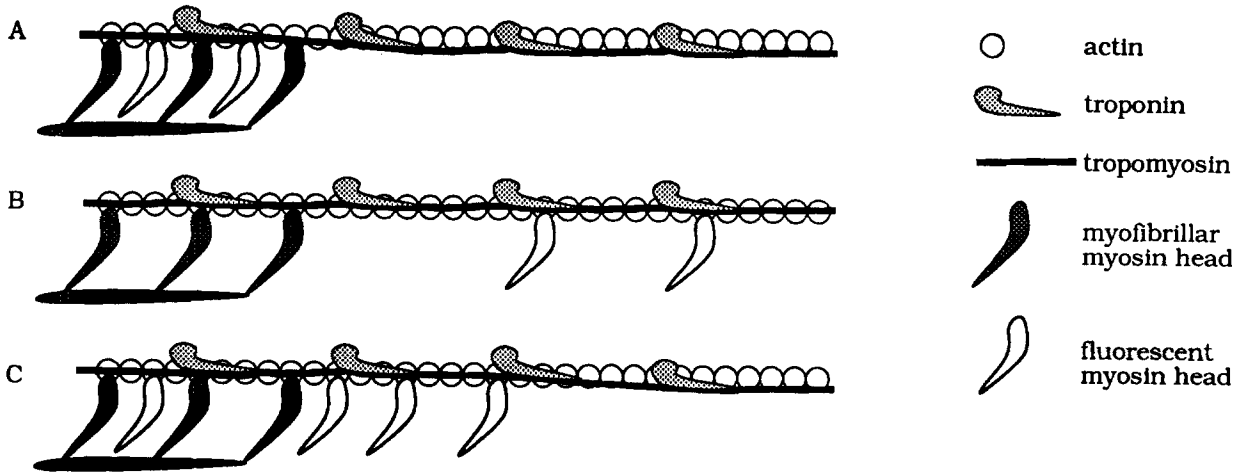

Figure 8. Molecular illustration for the binding of S1 to myofibrillar thin filaments near the A-band–I-band junction. The different molecules involved and their respective shapes are shown on the right of the figure. In A, the A/fS1 is high and calcium is absent, resulting in fluorescent myosin heads binding in the overlap region only. When calcium is present and the A/fS1 is high (*B*), the fluorescent myosin heads bind to actin in the I-band. At intermediate A/fS1 and in the absence of calcium (*C*), the fluorescent myosin heads bind mostly at the junction between the A-band and I-band and some bind in the overlap region. The illustration and the respective shapes and sizes of the molecules do not depict their true molecular dimensions.

staining Z-line was essentially the same as that observed in myofibrils stained in the presence of calcium.

Control Experiments

The staining patterns presented in Figs. 4 and 5 were obtained with R-X-M S1, taking advantage of this fluorophore's higher quantum yield and photostability. The same patterns at each A/fS1, with and without calcium, were observed when myofibrils were incubated with F-5-M S1. The similarity in the staining patterns obtained with both R-X-M and F-5-M S1 suggests that the fluorophore itself was not responsible for the staining patterns since these fluorophores differ substantially in structure and hydrophobicity. In other control experiments, the amount of fluorescent S1 was held constant but the A/fS1 was altered using unconjugated S1. The A/fS1 was maintained at 20:1 and an A/total S1 of 20:1, 10:1, and 7:1 was incubated with myofibrils. The patterns observed both with and without calcium as a function of A/total S1 were the same as those observed when conjugated S1 was used. More specifically, a bright band of fluorescence was observed at the I-band–A-band junction at intermediate A/total S1 in the absence of calcium, whereas the pattern was independent of A/total S1 in the presence of calcium. The conjugation efficiency was 30–50% (i.e., 0.3–0.5 mol fluorophore per mole S1). Assuming that the S1 was randomly labeled, the fS1 contained a mixture of 0:1, 1:1, and 2:1 mole fluorophore per mole S1. Thus, the actual staining patterns observed for fS1 were the result of a combination of the influences of both conjugated and unconjugated S1 upon the regulated thin filaments of the myofibril. These results suggest that neither the fluorophore nor the conjugation significantly influenced the ability of the troponin–tropomyosin complex to regulate the binding of S1 to actin. Also, experiments with rabbit psoas stained with either bovine cutaneous trunci S1 conjugates or rabbit white muscle S1 conjugates (prepared as described previously) gave the same results, suggesting that the S1 binding patterns are not specific for bovine muscle.

Double Staining in the Absence and Presence of Calcium

The images presented in Figs. 4 and 5 demonstrate that S1 binds at two different locations of actin sites in the sarcomere when myofibrils are incubated with S1 at high A/fS1, the location being dependent upon the absence or presence of calcium. To demonstrate these different sites within the same sarcomere, sequential double-staining experiments were undertaken; myofibrils were incubated with one fluorescent S1 in the absence of calcium and at high A:fS1, and were then incubated with the other fluorescent S1 in the presence of calcium and at the same A/fS1. An example of the binding patterns observed is shown in Fig. 6. The myofibrils were first incubated for 20 min with R-X-M S1 at 24 A:1 fS1 in the absence of calcium and were then pelleted and resuspended with fresh buffer containing F-5-M S1 at 24 A:1 fS1 in the presence of calcium. An aliquot was removed, fixed (within 30 s of initial mixing in the presence of calcium), and mounted. The S1 bound in the absence of calcium (Fig. 6, *b* and *e*) was located in the myofibrillar actin–myosin head overlap region, while S1 bound in the presence of calcium (Fig. 6, *c* and *f*) was found in the I-band (except at the Z-line). Shorter SL myofibrils (Fig. 6 *b*) gave calcium absent staining patterns with two fluorescent bands per sarcomere; the bands were wider and more closely spaced than those observed at longer SL (Fig. 6 *e*), supporting the overlap staining observed in the single label experiments. The S1 bound in the presence of calcium (Fig. 6, *c* and *f*) was distributed predominantly in the I-band. When the time of incubation with S1 in the presence of calcium was increased (Fig. 7), the pattern of the S1 bound in the absence of calcium became less distinct (compare Fig. 7, *b* and *e*, to Fig. 6, *b* and *e*). The S1 bound in the presence of calcium was essentially the same as that observed with minimal incubation time (compare Fig. 7, *c* and *f*, to Fig. 6, *c* and *f*). Prolonged incubation (i.e., >4 min) resulted in more of the S1, initially bound in the absence of calcium, being bound in the I-band. This demonstrated that the fS1 was slow to reequilibrate and bind

to other regions of the myofibril. The same patterns were observed as a function of calcium and time of incubation when R-X-M and F-5-M S1 were switched.

Discussion

Methods were developed for the conjugation of sulfhydryl-specific fluorophores to thiols other than SH_1 and SH_2 of S1, this procedure protecting the catalytic activity of the protein. A recent study showed that modification at SH_1 can result in the loss of the calcium sensitivity of the regulated actin-activated ATPase activity of myosin (Titus et al., 1989). Although we did not investigate the calcium-sensitive binding properties of S1 modified at SH_1, these properties of S1 modified at thiols other than SH_1 or SH_2 were investigated. Using these fluorescent probes, we have demonstrated several features of S1 binding to actin sites in the myofibril including the calcium sensitivity of the location of binding, and the concentration dependency and cooperativity of binding in the absence of calcium.

The binding of fluorescent conjugates of the myosin head to myofibrils has been studied by several researchers (Aronson, 1965; Borejdo and Assulin, 1980; Herman and Pollard, 1978; Sanger, 1975; Sanger et al., 1984). None, however, demonstrated the calcium sensitivity of the location of binding. By using different fluorescent conjugates of S1 and controlling the ratio of myofibrillar actin to fluorescent S1, we have demonstrated that the binding of S1 to myofibrillar actin is sensitive to the status of tropomyosin–troponin on the thin filaments. The actin sites within the myofibril can be either occupied (by myofibrillar myosin heads) or unoccupied, and these latter sites can have either a high affinity (activated) or a low affinity (inactivated) for S1 as modeled for reconstituted thin filaments (Hill et al., 1980, 1983). The affinity of the actin site for S1 is determined by the status of the tropomyosin–troponin complex. In the absence of calcium, the unoccupied sites within a myofibril are likely a mixture of high and low affinity sites, while in the presence of calcium, the sites are all high affinity. Suspension of myofibrils without calcium in a dilute solution of fluorescent S1, subsaturating with respect to total myofibrillar actin, should result in the high affinity sites becoming occupied by fluorescent S1. These sites may be in the less accessible overlap region or in the more accessible I-band region. The location or region of the myofibril that contains the fluorescence represents the portion of the myofibrillar thin filament that contains these high affinity sites. In the absence of calcium and at the lowest S1 concentration used (24 A:1 S1), the conjugate bound solely to the portion of the thin filaments in the overlap region of the myofibril, while in the presence of calcium it bound predominantly in the nonoverlap region of the thin filaments (I-band), with much less binding in the overlap region and no binding at the Z-line. Binding in the overlap region in the absence of calcium is explained by the presence of rigor complexes formed by myofibrillar myosin heads in the region of overlap; these rigor complexes altered the thin filament such that actin sites within the functional unit were activated (high affinity), while those sites within the I-band were not activated (low affinity). This calcium-independent mechanism for activation of the thin filament has been demonstrated in (*a*) muscle fibers by x-ray diffraction (Haselgrove, 1972), changes in fluorescence of chemically modified troponin-C (Güth and Potter, 1987), and tension generation at low ATP concentration (Godt, 1974; Moss and Haworth, 1984; Reuben et al., 1971; White, 1970); (*b*) myofibrils by measuring the ATPase activity at low ATP concentration (Weber, 1970); and (*c*) in reconstituted regulated actin–myosin head systems by measuring ATPase activity at low ATP concentration (Bremel and Weber, 1972; Bremel et al., 1972), the kinetics of S1 binding (Trybus and Taylor, 1980), and the equilibrium binding of S1 to regulated thin filaments (Greene, 1982; Greene and Eisenberg, 1980).

Studies using regulated thin filaments and S1 suggest that, at actin/S1 <10:1, S1 binding occurs in the absence of calcium while at higher ratios calcium-sensitive binding can be demonstrated (Murray et al., 1980). In the overlap region of the thin filaments in the myofibril, the ratio of actin monomers to myosin heads is between 1.7:1 and 2.0:1 (Squire, 1981). Biochemical studies suggest that all myofibrillar heads are bound to myofibrillar actin in the region of overlap under rigor conditions. (Lovell and Harrington, 1981; Cooke and Franks, 1980). The actin/S1 for activation and the approximate ratio of actin monomers to bound myofibril myosin head in the overlap region favor activation of the thin filament in this region of the myofibril. This mechanism of activation has been called rigor activation (Bremel and Weber, 1972). At 24 A:1 fS1, the soluble heads did not activate actin sites in the I-band, since negligible binding was observed in the nonoverlap region of the thin filaments. However, in the presence of calcium, binding was observed predominantly in the nonoverlap region of the thin filaments. In this system, calcium binding to troponin resulted in activation of all accessible actin sites in the thin filament.

Cooperative activation of the thin filament (transition from low affinity to high affinity) by soluble myosin heads in the absence of calcium was apparent when the concentration of S1 was increased (A/fS1 decreased). Studies using S1 and regulated thin filaments demonstrate the sigmoidal nature of the equilibrium-binding isotherm (Greene and Eisenberg, 1980; Greene, 1982) and the biphasic character of the kinetics of S1 binding to regulated actin in the absence of calcium (Trybus and Taylor, 1980). These data have been interpreted as demonstrating positive cooperativity for the binding of S1 to regulated actin. The current study visually demonstrates this cooperative binding process since, at intermediate A/fS1, S1 bound predominantly to the region of the thin filaments adjacent to the overlap region, this being seen as a narrow band of bright fluorescence at the edge of the A-I junction in the myofibril (Fig. 4, *c* and *e*). On either side of this band in the half sarcomere, the fluorescence was less: that on the A-band side probably because of fewer sites (myofibrillar myosin heads occupied about half of the actin sites), and that on the I-band side (close to the Z-line) because the actin sites were inactivated. The observation of nonrandom binding of S1 (in the I-band) in the absence of calcium at intermediate A/fS1 suggests that a cooperative binding process was involved. Random binding was demonstrated in myofibrils stained at the same A/fS1 but in the presence of calcium, a condition that dramatically decreases cooperative binding of S1 to thin filaments (Greene, 1982; Greene and Eisenberg, 1980). These patterns show available actin sites that were activated by the binding of calcium to troponin. The change in the S1-binding pattern as the A/fS1 was decreased in the absence of calcium suggests that the

thin filament is activated from the region of overlap towards the Z-line (i.e., the bright band at the A-I junction becomes wider towards the Z-line as the A/fS1 is decreased from 16:1 to 4:1). This change in the S1-binding pattern likely involves end-to-end tropomyosin–tropomyosin interactions on the thin filament. The mathematical models for S1 binding to regulated thin filaments incorporate tropomyosin interactions (Hill et al., 1980, 1983), and the effect of this end-to-end interaction has been investigated experimentally using COOH-terminal-truncated tropomyosin (Walsh et al., 1986; Pan et al., 1989). Enzymatic studies have shown that tropomyosin interactions have little influence on the ATPase activity as a function of calcium concentration when very high A/S1 are used (Walsh et al., 1986), whereas equilibrium-binding studies have shown a dramatic influence of tropomyosin interaction on the binding of S1–ADP (S1 with bound ADP) to regulated thin filaments in the absence of calcium.

The patterns of binding that were observed are illustrated in Fig. 8. At high A/fS1 and in the absence of calcium, the fluorescent heads bind to the actin sites between existing rigor complexes formed by myofibrillar myosin heads (Fig. 8 *A*), whereas in the presence of calcium the fluorescent heads bind sites in the I-band (Fig. 8 *B*). The fluorescent heads are distributed in a different pattern in the absence of calcium when the A/fS1 is decreased (Fig. 8 *C*); they bind predominantly to actin sites in the I-band near to the A-band. The illustration demonstrates different hypothetical positions for the tropomyosin–troponin complex along the actin filament under the different conditions used for staining. The patterns of binding modeled in Fig. 8, *A* and *B*, were shown within the same myofibril when a sequential double-staining procedure was employed (Figs. 6 and 7). In these myofibrils, two mechanisms were involved in converting blocked sites to unblocked sites: rigor activation of the sites within the overlap region, and calcium activation of the sites within the I-band. In preliminary double-staining experiments at 24 A:1 fS1, the protein was bound in the presence of calcium followed by binding S1 in the absence of calcium. The staining patterns were independent of calcium using this sequential-staining protocol. This result may be due to the S1, bound in the presence of calcium (and thus randomly distributed on the unoccupied actin sites), maintaining the other unoccupied sites in the activated state in the absence of calcium. This would give the same staining pattern for the different incubation conditions.

These studies visually demonstrate that the actin sites within the I-band of the myofibril bind S1 only when calcium is present, or when the amount of S1 is high, a result directly supporting calcium regulation of S1 binding and cooperative binding in the absence of calcium. The patterns suggest that S1 binds actin sites nearest to an existing rigor complex, whether that complex was formed from myofibrillar myosin heads or from S1. In relaxed muscle, the amount of nucleotide-free myosin is negligible so the ratio of actin to nucleotide-free myosin is very high; the thin filaments are not activated by myosin heads and the steric hindrance model applies. In activated muscle, the rigor complex is a short-lived state in the acto-myosin ATPase cycle (Goldman, 1987), but it may be involved in activating the thin filament. Studies with *N*-ethylmaleimide–modified S1, which forms an irreversible rigor complex, showed that maximal ATPase activity occurred when the actin sites were partially saturated with rigor complexes in the presence of calcium (Greene et al., 1987; Nagashima and Asukura, 1982; Williams et al., 1988). Other studies showed that structural changes in tropomyosin, measured from a fluorescence probe covalently attached to tropomyosin, required both calcium and partial saturation of the actin sites with S1 (Ishii and Lehrer, 1987). These results suggest that rigor complexes further activate the thin filament even in the presence of calcium. A corollary of this observation is that these complexes increase the calcium sensitivity of thin filaments. This was shown by Godt (1974), who demonstrated that the calcium sensitivity of tension in skinned fibers was increased as the concentration of ATP was decreased.

Chalovich and Eisenberg (1982) tested the steric-blocking hypothesis from a kinetic standpoint by measuring the amount of S1 bound to regulated actin during steady-state ATPase activity. They found that the binding of S1 to actin was low and independent of calcium, leading them to conclude that troponin–tropomyosin does not block the binding of the major kinetic intermediates of S1 to actin during steady-state ATPase activity and that the regulatory proteins appear to inhibit a kinetic step in the ATPase cycle. Rosenfeld and Taylor (1987*a*,*b*) concluded from their kinetic studies that either a partial steric-blocking mechanism or a nonsteric-blocking, conformational mechanism may be responsible for regulation of actomyosin ATPase activity. They also suggested that the step regulated by calcium is the transition from a weakly bound complex to a strongly bound complex. The data presented in the current paper demonstrate regulation of the location of binding of a strongly bound complex; they do not, however, directly delineate the molecular mechanism(s) that regulate contraction.

The observations from the present study have ramifications on binding studies using reconstituted regulated thin filaments. At high A/S1, the S1 may all be bound to a confined region of the actin filament, this region being highly saturated while other regions may be essentially devoid of bound S1. Quantitative structural studies at the level of the regulated thin filament are needed to extend our understanding of the interactions between S1 binding to the thin filament and regulation of this process by the tropomyosin–troponin complex.

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