Thin Filament Activation Probed by Fluorescence of *N*-((2-(Iodoacetoxy)ethyl)-*N*-methyl)amino-7-nitrobenz-2-oxa-1,3-diazole-Labeled Troponin I Incorporated into Skinned Fibers of Rabbit Psoas Muscle

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ABSTRACT A method is described for the exchange of native troponin of single rabbit psoas muscle fibers for externally applied troponin complexes without detectable impairment of functional properties of the skinned fibers. This approach is used to exchange native troponin for rabbit skeletal troponin with a fluorescent label (*N*-((2-(iodoacetoxy)ethyl)-*N*-methyl) amino-7-nitrobenz-2-oxa-1,3-diazole, IANBD) on Cys^{133} of the troponin I subunit. IANBD-labeled troponin I has previously been used in solution studies as an indicator for the state of activation of reconstituted actin filaments (Trybus and Taylor, 1980. *Proc. Natl. Acad. Sci. USA.* 77:7209–7213). In the skinned fibers, the fluorescence of this probe is unaffected when cross-bridges in their weak binding states attach to actin filaments but decreases either upon the addition of Ca^{2+} or when cross-bridges in their strong binding states attach to actin. Maximum reduction is observed when Ca^{2+} is raised to saturating concentrations. Additional attachment of cross-bridges in strong binding states gives no further reduction of fluorescence. Attachment of cross-bridges in strong binding states alone (low Ca^{2+} concentration) gives only about half of the maximum reduction seen with the addition of calcium. This illustrates that fluorescence of IANBD-labeled troponin I can be used to evaluate thin filament activation, as previously introduced for solution studies. In addition, at nonsaturating Ca^{2+} concentrations IANBD fluorescence can be used for straightforward classification of states of the myosin head as weak binding (nonactivating) and strong binding (activating), irrespective of ionic strength or other experimental conditions. Furthermore, the approach presented here not only can be used as a means of exchanging native skeletal troponin and its subunits for a variety of fluorescently labeled or mutant troponin subunits, but also allows the exchange of native skeletal troponin for cardiac troponin.

INTRODUCTION

Muscle contraction is thought to result from a cyclic interaction of the myosin heads with actin filaments. During this cyclic action, the myosin heads alternate between two classes of states (for reviews cf. Taylor, 1979; Eisenberg and Hill, 1985; Hibberd and Trentham, 1986; Brenner, 1990). One class, in which cross-bridges make little contribution to force generation, is characterized by low actin affinity and little or no ability to activate thin filaments (cf. Chalovich et al., 1983). The second class includes the main force-generating states and is characterized by the potential to bind to actin with high affinity and to activate the thin filament. The ability to activate the thin filament is a particularly useful criterion for distinguishing the two classes of states of the myosin head because this ability is largely independent of the absolute binding affinity of the myosin head for actin and therefore largely independent of ionic strength (Chalovich et al., 1983). Instead, the potential to enhance activation of the thin filament upon attachment of myosin heads appears to depend on the difference in affinity of the myosin head for the inactive versus active forms of the actin filament, as discussed by Greene and Eisenberg (1980) and Hill et al. (1981). One challenge in understanding the molecular mechanisms of muscle contraction is to determine how regulation of the thin filament, i.e., Ca^{2+}-controlled movement of tropomyosin on the surface of the actin filaments, affects the interaction of both weak and strong binding states of the myosin head with the regulated thin filament.

Possible approaches to these questions are the use of fluorescent probes attached to regulatory proteins to monitor the state of the thin filament and the use of mutants of regulatory proteins generated by site-directed mutagenesis and expressed and purified from suitable expression systems. Both approaches require extensive exchange of native regulatory proteins, e.g., troponin and its subunits, for externally applied fluorescently labeled or mutant proteins with as little change as possible in the properties of the skinned fibers.

Previously, protocols have been developed for the exchange of troponin C and troponin I by extraction, followed by reconstitution with an externally applied protein (e.g., Zot and Potter, 1982; Moss et al., 1985; Babu et al., 1986; Nakayama et al., 1990; Hatakenaka and Ohtsuki, 1991; Strauss et al., 1992; Rarick et al., 1997). We set out to

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develop an approach that permits the introduction of any troponin subunit or combination of troponin subunits into single rabbit psoas muscle fibers while maintaining normal fiber function. The approach used here is one of exchange rather than extraction followed by reconstitution. This approach follows other observations that exchange processes may be more efficient than protocols based on extraction and subsequent reconstitution. For instance, we had observed that M-line-associated, fluorescently labeled creatine phosphokinase (CPK) is only slowly extracted (lost) from skinned fibers under physiological and near-physiological conditions, but is rapidly replaced (chased) when, under the same conditions, skinned fibers are exposed to externally applied, unlabeled CPK (Kraft et al., 1995b). This suggested that molecules that can reversibly dissociate from their binding sites can be replaced by externally applied molecules that compete for the free binding sites.

Here we report the general approach to the exchange of whole troponin and demonstrate its validity by introducing *N*-((2-(iodoacetoxy)ethyl)-*N*-methyl)amino-7-nitrobenz-2-oxa-1,3-diazole (IANBD)-labeled troponin I (TnI) into skinned fibers of the rabbit psoas muscle. This probe was previously used in solution studies to follow the state of thin filament activation (Trybus and Taylor, 1980). The method for the exchange of troponin, described here, is similar to that described for the exchange of whole troponin in myofibrils (Shiraishi et al., 1992). We show that the fluorescence of IANBD-labeled TnI can be used for straightforward classification of different states of the myosin head, generated by the presence of different nucleotides or nucleotide analogs. A preliminary account of this work was previously presented (Chalovich and Brenner, 1995; Brenner et al., 1998).

MATERIALS AND METHODS

Preparation of proteins

Myosin (Kielley and Harrington, 1960), S1 (Weeds and Taylor, 1975), actin (Eisenberg and Kielley, 1974), and tropomyosin-troponin (Eisenberg and Kielley, 1974) were prepared from the back muscle of rabbits. Tropomyosin was separated from troponin by hydroxyapatite chromatography (Eisenberg and Kielley, 1974). The troponin was further purified by chromatography on Cibacron blue, as described by Reisler et al. (1980). Bovine cardiac troponin was prepared as described by Potter (1982).

Preparation of troponin with TnI modified at Cys^{133}

The troponin-tropomyosin complex was reduced at 30°C for 1 h in a solution containing 100 mM KCl, 20 mM phosphate buffer (pH 6.5), 1 mM EDTA, and 2 mM fresh dithiothreitol. After exhaustive dialysis against 30 mM KCl, 10 mM imidazole, 1 mM $MgCl_2$, 1 mM EGTA, 0.1 mM dithiothreitol, the sulfhydryl group of TnI was modified. Troponin-NBD was prepared by reaction of a 1 mg/ml solution of tropomyosin-troponin with a fivefold molar excess of IANBD (Molecular Probes, Eugene, OR) in *N*,*N*-dimethylformamide. The mixture was incubated in the dark at 4°C for 4 h, and the reaction was stopped with an excess of dithiothreitol. Excess probe was removed by repeated dialysis. The same procedure was used for the preparation of rhodamine-labeled troponin I, except that the whole reduced troponin was treated with a fivefold molar excess of rhodamine X-iodoacetamide (Molecular Probes). A slight amount of labeling of tropomyosin occurred under this condition. However, subsequent to fluorescent labeling, the troponin complex was isolated by hydroxyapatite chromatography such that no tropomyosin was present in the final product. A small amount of label was also incorporated into troponin C and troponin T in some preparations. The labeling of these two subunits varied from an undetectable level up to some 9% of the incorporation found in troponin I.

Fiber preparation

Skinned fibers of rabbit psoas muscle were isolated and chemically skinned with Triton X-100 as previously described (Brenner, 1983; Yu and Brenner, 1989), but with the recent modification of adding several protease inhibitors (for details see Kraft et al., 1995a). Single fibers were isolated as previously described (Yu and Brenner, 1989), but within a few hours after dissection of fiber bundles from the rabbit. Isolated single fibers were kept for up to 5 days without a detectable loss of sarcomeric proteins, but with the advantage of much improved diffusion of substrate and products. Some experiments were performed with fibers isolated from fiber bundles that had been frozen in liquid propane and stored in liquid nitrogen according to our previously described procedures (Kraft et al., 1995b). No detectable differences were found compared to fibers that were not subject to this freezing, storage, and thawing procedure.

Solutions

All solutions were adjusted to pH 7.0 at the experimental temperature (5°C). Chemicals were obtained from Sigma Chemie München, FRG, except when noted otherwise.

Skinning solution contained (in mM) 5.0 KH_2PO_4; 3.0 Mg-acetate; 5.0 EGTA; 1.0 Na_2ATP (Merck, Darmstadt, Germany); 50 Na-creatine phosphate; 5.0 NaN_3; 10 glutathione; 2.0 dithiothreitol; 0.1 4-(2-aminoethyl)-benzenesulfonyl fluoride (Calbiochem-Novabiochem GmbH, Bad Soden, Germany); 0.01 each of leupeptin, antipain, E64, and pepstatin; and 1 mg/ml aprotinin.

Preactivating and activating solutions contained (in mM) 10 imidazole, 2.0 $MgCl_2$, 1.0 MgATP, 1.0–3.0 EGTA (or CaEGTA), 10 caffeine, and 500 U/ml (Sigma definition of units) of creatine kinase (Sigma, München, Germany). Ionic strength was adjusted by adding the appropriate amount of sodium creatine phosphate, assuming a contribution to ionic strength of 3 mM/ mM of sodium creatine phosphate. The minimum concentration of added creatine phosphate was 10 mM, resulting in 50 mM as the lowest ionic strength for activation experiments.

Solutions of different pCa were obtained by mixing appropriate volumes of preactivating and activating solutions.

Standard relaxing solution contained (in mM) 10 imidazole, 2.0 $MgCl_2$, 1.0 MgATP, and 1.0 EGTA, resulting in a minimum ionic strength of ~20 mM when no creatine phosphate was added.

Prerigor solution contained (in mM) 10 imidazole, 2.5 EGTA, and 15 EDTA.

Rigor solution was composed of (in mM) 10 imidazole, 2.5 EGTA, and 2.5 EDTA.

Mg-pyrophosphate ($MgPP_i$) solution contained (in mM) 10 imidazole, 4.0 $MgPP_i$, 2.0 $MgCl_2$, 1.0 CaEGTA, and 0.2 Ap_5A.

MgATPγS-solution contained (in mM) 10 imidazole, 10 MgATPγS, 2.0 $MgCl_2$, 3.0 EGTA, 200 glucose, 0.5 U/ml hexokinase, and 0.2 Ap_5A.

For all solutions, except skinning solution and preactivating and activating solutions, ionic strength was adjusted by adding appropriate amounts of potassium propionate.

Exchange buffer contained (in mM) 20 3-(*N*-morpholino)propanesulfonic acid, 5 $MgCl_2$, 5 EGTA, 240 KCl, 5 dithiothreitol, and 7 μg/ml pepstatin (pH 6.5) at 20°C or 5°C.

Quantification of troponin exchange

The exchange of intrinsic skeletal muscle troponin of skinned fibers for cardiac troponin was determined by gel electrophoresis and Western blot analysis. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was done either with the PhastSystem (Pharmacia Fine Chemicals, Uppsala, Sweden), using a 8–25% gradient of polyacrylamide, or on minigels (Hoefer, San Francisco, CA), using 12% polyacrylamide gels. The gels were stained with Coomassie brilliant blue. In some instances, the electrophoresed proteins were blotted for 1 h at 24 V onto Immobilon (Millipore, Bedford, MA) membranes, using a semidry blotter with the buffer system of Towbin et al. (1979). The membranes were subsequently blocked (5% powdered milk in Tris-buffered saline containing 0.01% Tween 20) and then incubated with a mixture of monoclonal anti-α-actin (Sigma Chemical Co., St. Louis, MO) and monoclonal anti-skeletal troponin I antibodies (Biodesign International, Kennebunk, ME). After washing and incubation with the second antibody, the actin and skeletal troponin I were visualized using enhanced chemiluminescence (Mathews et al., 1985).

Experimental setup

The apparatus to record mechanical and fluorescent signals from segments of skinned fibers was developed from the previously described experimental setup (Brenner, 1980; Brenner and Eisenberg, 1986). The setup was fitted with a mercury arc lamp and an interference filter (Scientific Instruments, Heidelberg, Germany) to select the proper wavelength for excitation of IANBD fluorescence (486.1 nm, 11 nm bandwidth; Melles Griot, Irvine, CA). The light of the mercury arc lamp was focused on the skinned fiber by means of two cylindrical lenses to maximize the intensity of the exciting light at the specimen. The emitted light was passed through a OG530 filter with 50% transmission at 530 nm (Melles Griot). The binocular phototube of a home-built inverted microscope could be replaced with an optical device that imaged a section of the fiber on a photomultiplier tube (Scientific Instruments, Heidelberg, Germany). The voltage of the photomultiplier tube was set to obtain optimal signal-to-noise ratio, usually to some 1000 V. The bandwidth (−3 dB) of the detection system was 2.4 kHz, with a time constant of 80 μs for the response to a step change in the intensity output by 5 V, which is the upper limit for maximum fluorescence changes.

Confocal microscopy

Equilibration of skinned fibers with externally applied troponin was followed by confocal microscopy to estimate the time required to reach homogeneous troponin exchange across the fiber and to characterize the labeling pattern within the sarcomeres. Because of the available laser source for the excitation of fluorescence, we had to use rhodamine-labeled troponin (Rh-TnI) instead of the IANBD-labeled troponin. To identify the different structures within the sarcomeres, the M-line was selectively stained by an antimyomesin antibody and a FITC-labeled secondary antibody.

The system for confocal microscopy was previously described (Kraft et al., 1995b) and consisted of a Zeiss Axiophot fluorescence microscope, a Biorad MRC-600 confocal scanner, and a Silicon Graphics Personal Iris 4D/25 workstation. The system was equipped with an argon/krypton mixed gas laser. The images were recorded using a Zeiss Planapochromat 40 × NA 1.0 oil immersion objective. Image processing was done on the Silicon Graphics work station, using Imaris (Bitplane AG, Zürich, Switzerland).

To follow equilibration by confocal microscopy, fibers were mounted in a shallow slot-shaped chamber built on a microscope slide and closed with a coverslip (cf. Kraft et al., 1995b).

The time course for the equilibration of fibers with troponin with the TnI subunit labeled with rhodamine was quantitated as previously described (Kraft et al., 1995b), e.g., by taking the ratio of intensity at superficial areas over intensity in the fiber core seen in longitudinal (optical) sections through the center of the fibers. The time course of fluorescence decay as a measure of reexchange of rhodamine-labeled troponin (rhodamine-labeled TnI subunit) for non-rhodamine-labeled troponin was followed in a similar way; longitudinal (optical) sections through the center of the fibers were recorded (cf. Fig. 1), and intensity profiles along a line perpendicular to the fiber axis (cf. *white line* in the micrograph of Fig. 1) were plotted. To characterize the time course of reexchange, the total integrated intensity of these profiles was plotted against time (cf. Fig. 3 *A*).

Mechanical measurements

Isometric force and force redevelopment were recorded as previously described (Brenner and Eisenberg, 1986). In short, after a period of 100–200 ms of unloaded or lightly loaded shortening, fibers were restretched to their isometric sarcomere length to restore initial isometric filament overlap. Fibers were then held at a fixed sarcomere length as tension redeveloped from a low initial level of ~10% of the isometric steady-state force (cf. Brenner 1985).

X-ray diffraction experiments

Equatorial x-ray diffraction patterns were recorded by using the previously described laboratory setup (Brenner et al., 1984). Integrated intensity, full width at half-maximum, and position of the two innermost reflections (1,0 and 1,1 reflections) were determined after a linear background stripping as previously described (Brenner et al., 1984). Exchange of native troponin for Rh-labeled troponin (Rh-TnI) was performed according to the standard protocol (see below) while the fibers were kept in the x-ray chamber.

Experimental protocol

Skinned fibers were attached to the force transducer and lever system of the experimental setup, using a cyanoacrylate glue (Histoacryl; B. Braun Surgical GmbH, Melsungen, Germany). The ends of the fibers were fixed

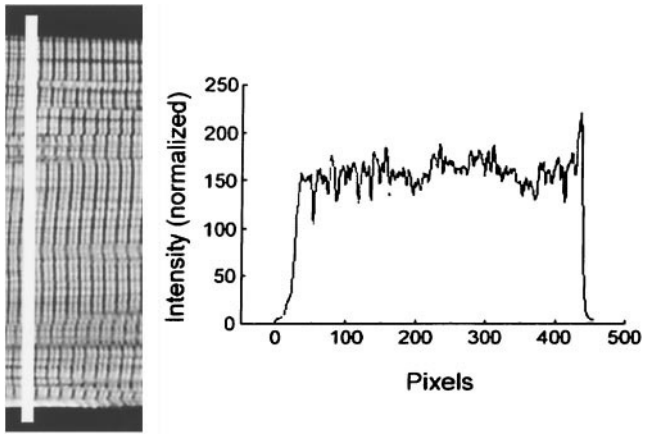

FIGURE 1 Experimental approach to characterizing the change in fluorescence when fluorescently labeled troponin (Rh-labeled troponin I subunit) is "chased" with troponin that is "invisible," with the rhodamine excitation-emission system of the confocal laser scanning microscope (e.g., troponin with IANBD-labeled troponin I subunit). (*Left*) Segment of a longitudinal optical section through the center of a skinned fiber. (*Right*) Intensity profile recorded along the line across the fiber, as indicated by white line in the segment of the longitudinal section. To obtain this profile, 10 profiles within the area of the white line, each one pixel wide, were averaged to reduce intensity fluctuations due to cutting through different parts of the sarcomeres and thus through different labeling densities. The total area under such profiles was determined and plotted versus time to follow the loss of fluorescently labeled troponin over time.

with glutaraldehyde according to the method of Chase and Kushmerick (1988), with some modifications (Kraft et al., 1995a).

After mounting and setting the sarcomere length at 2.3–2.4 μm, isometric force and force redevelopment were recorded under various levels of Ca^{2+} activation. Subsequently, TnI was exchanged for IANBD-labeled TnI by exchange of the whole troponin complex. For the exchange process, fibers were put into rigor via prerigor solution (Brenner et al., 1982, 1986) at 5°C. After several thorough washes to remove traces of ATP, fibers were transferred into exchange buffer containing 0.3–1.5 mg/ml of skeletal troponin with the IANBD-labeled TnI (unless otherwise stated), and the temperature was raised to 20°C. In some experiments, the temperature for exchange was kept at 5°C, resulting in no detectable difference compared with exchange at higher temperature, except for the need for a somewhat longer time for reaching maximum exchange. After exchange periods ranging from 2 to 12 h, unbound troponin was removed by washing the fibers in several changes of exchange buffer without IANBD-labeled troponin for at least 30 min. After this washing period, IANBD fluorescence and mechanical parameters were recorded under the various experimental conditions. During Ca^{2+} activation fibers were permanently cycled between isometric steady-state contraction and short (150–200 ms) periods of lightly loaded or unloaded shortening with subsequent restretch to the initial (isometric) sarcomere length. Repeating this maneuver every 5 s stabilized the striation pattern during prolonged activation (Brenner, 1983).

RESULTS

Exchange of native troponin for troponin with fluorescently labeled TnI

Exchange studied by PAGE

Quantification of the exchange of native troponin for externally applied fluorescently labeled troponin was achieved by PAGE and Western blot analysis. However, to differentiate native material from exchanged material, we had to use cardiac troponin for exchange. Both cardiac troponin I (TnI) and troponin T (TnT) are larger than their skeletal counterparts (Hatakenaka and Ohtsuki, 1991), such that TnI and TnT of both sources can be separated by SDS-PAGE. Fig. 2 *A* shows that under several conditions of exchange (varied length of exchange time and troponin concentration) a high level of exchange can be achieved. Fig. 2 *A* (*lanes a* and *b*) shows the presence of cardiac troponins I (cTnI) and T (cTnT) in the exchanged fibers. Troponin C was not resolved well enough to confirm that this component is also exchanged. However, other data indicate the exchange of troponin C by this method. For instance, the force-pCa relation of rabbit psoas fibers changed to that characteristic of cardiac troponin C when the exchange was made with bovine cardiac troponin (cf. Fig. 2 *B*). Because skeletal TnI (sTnI) comigrates with light chain 1 (LC1), it is unclear whether sTnI is still present after the "exchange" procedure. Lanes c and d, however, show that after incubation for 6 h with 3 mg/ml of cardiac troponin, the original skeletal troponin I is no longer detectable by Western blot analysis. Yet fibers are still fully Ca^{2+}-regulated; that is, they are fully relaxed at low Ca^{2+} and generate tension when Ca^{2+} is raised (cf. Fig. 2 *B*). This indicates that there is real exchange and not simply adsorption of additional troponin. Lanes e–h demonstrate that troponin T is also exchanged by this method. Lanes e and f were run in the absence of urea.

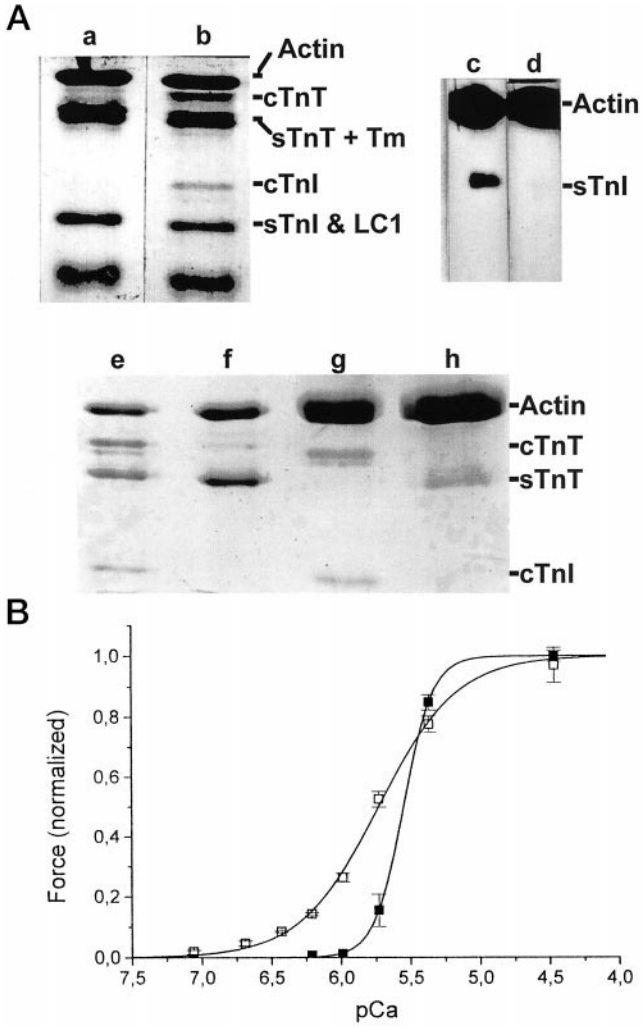

FIGURE 2 (*A*) SDS-polyacrylamide gels of fibers after the exchange of endogenous troponin for cardiac troponin under different conditions. Only those parts of the gels containing troponin are shown. *Lanes a, b, c*, and *d* were run to emphasize the exchange of cTnI for sTnI. *Lanes a* (control) and *b* (3 h of exchange with 0.5 mg/ml cardiac troponin, 5°C) are from 12% polyacrylamide gels stained with Coomassie blue. *Lanes c* (control) and *d* (6 h of exchange with 3 mg/ml cardiac troponin, 5°C) are Western blots of 10% polyacrylamide gels probed with an antibody selective for sTnI. *Lanes e, f, g*, and *h* were run to emphasize that sTnT also is exchanged for cTnT. The fibers were incubated with 2.5 mg/ml cardiac troponin for 17 h at 5°C. *Lanes e* and *g* are from exchanged fibers, and *f* and *h* are controls. *Lanes e* and *f* were run in normal fashion, and the tropomyosin co-migrated with the sTnT. *Lanes g* and *h* were run in a buffer containing 8 M urea in addition to the SDS. In that case, the tropomyosin comigrated with the actin, and the sTnT can be seen more readily. (*B*) Force-pCa relations recorded before (■) and after (□) 8 h of incubation in exchange buffer with 2.5 mg/ml of cardiac troponin. Note the much shallower force-pCa relation after incubation. This is similar to the change reported upon partial extraction of TnC from skeletal fibers followed by substitution with cardiac TnC (Moss et al., 1986).

Under these conditions, the skeletal troponin T comigrates with tropomyosin. While the addition of cTnT to the fibers can be seen (Fig. 2 *A*, *lanes e* and *g*), it is not clear how much of the skeletal troponin T had been removed. Therefore, lanes g and h were run in the presence of 8 M urea in the sample. Under these conditions, tropomyosin comi-

grates with actin. It can be seen that skeletal TnT is no longer present in the exchanged fibers (*lane g*). This is further evidence for true exchange and shows that at 5°C within 6 h of incubation with 3 mg/ml of cardiac troponin, exchange for cardiac troponin is essentially complete, as confirmed after 17 h of incubation at 5°C with 2.5 mg/ml. It should be noted that the use of cardiac troponin instead of skeletal troponin may have required longer times to achieve maximum exchange than necessary for skeletal troponin.

Confocal microscopy

To follow exchange in real time, using skeletal troponin instead of the cardiac troponin, and to examine the local distribution of exchanged troponin within the fiber cross section and within the sarcomeres, we used confocal laser scanning microscopy. We had to use skeletal troponin with rhodamine-labeled TnI because no suitable laser source was available to excite the IANBD fluorescence in the confocal laser scanning microscope.

The time course of equilibration of fibers with fluorescently labeled troponin and the labeling pattern at the sarcomere level are shown in Fig. 3. In Fig. 3 *A* three longitudinal optical sections through the core of a fiber are shown that were recorded after 7, 37, and 260 min of incubation with 0.3 mg/ml of Rh-TnI-containing troponin. Note that the images after 7 and 37 min show lower intensity in the core of the fiber. This is no longer present in the third image

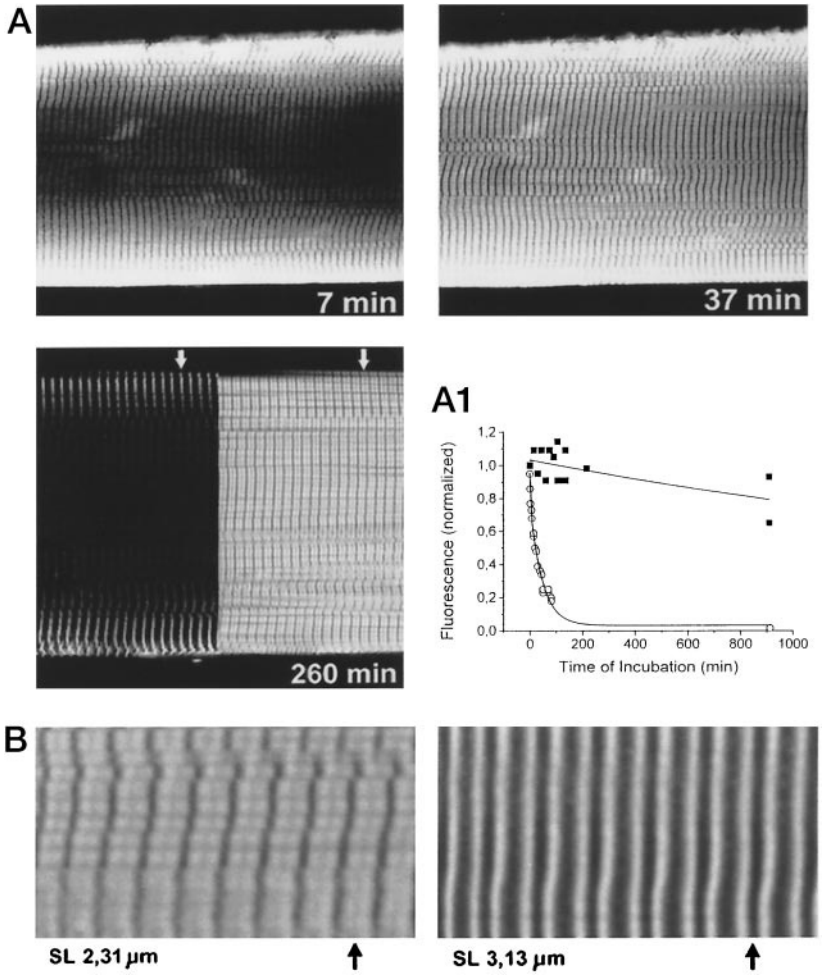

FIGURE 3 Labeling pattern and time course of equilibration followed by confocal microscopy. (*A*) Time course of equilibration with fluorescently labeled troponin (Rh-troponin-I) after 7, 37, and 260 min of incubation in exchange buffer with 0.3 mg/ml Rh-labeled troponin. To identify the M-line within the sarcomeres, the image taken after 260 min shows the fluorescence of Rh-labeled troponin I (*right*) and the fluorescence of a FITC-labeled antimyomesin antibody (*left*; cf. Kraft et al., 1995b). Corresponding M-line positions are indicated by arrows. *A*1: Chase of fluorescently labeled troponin (rhodamine-labeled troponin I subunit) by incubation in exchange buffer with 0.3 mg/ml of IANBD-labeled troponin (○). To achieve preexchange of native troponin for Rh-TnI-troponin, the fiber was incubated for 2.5 h in 0.3 mg/ml troponin with rhodamine-labeled TnI. Upon incubation with exchange buffer containing the IANBD-labeled troponin, the Rh fluorescence changed with a half-time of ~20 min. Control after 12 h gives the final lower limit of fluorescence (background level). Incubation in exchange buffer without any troponin results in only marginal loss of fluorescence over the same 12-h period (■). Solid lines are fits of exponential functions to the data points (modified from Kraft et al., 1995c). (*B*) The labeling pattern for short and long sarcomere lengths. The sarcomere length of the two fibers was first set at 2.31 and 3.13 µm, respectively. Then exchange was initiated by incubation for 2 h in exchange buffer with 0.3 mg/ml of rhodamine-labeled troponin (Rh-labeled TnI subunit). Arrows indicate the M-line, as identified by FITC-labeled antimyomesin antibody (cf. *a*). Note that fluorescence labeling 1) is highest in the area that correlates with overlap between actin and myosin filaments, 2) is reduced in the I-band part of the actin filaments, and 3) at long sarcomere lengths appears even lower in the H-zone.

recorded after 260 min, indicating still incomplete equilibration after 37 min. In the third image both fluorescence of Rh-labeled TnI and that of a FITC-labeled secondary antibody against an antimyomesin antibody are shown to identify the M-lines (equivalent structures are indicated by *arrows*).

To better establish the time required for the exchange of troponin, we examined the time course of reexchanging the fluorescently labeled TnI for unlabeled TnI, because it is easier to detect the approach to background-level intensity rather than to a maximum that is difficult to predict because it varies, e.g., with the geometry of the experimental arrangement, like fiber diameter and thus the position of the optical section within the fiber. This is because fibers are thick specimens (on the order of 100 μm), such that absorption of exciting and emitted light within the fiber and defocusing due to optical aberrations and inhomogeneities are significant. It is therefore expected that maximum intensity in an optical section through the core of a fiber will vary, depending on fiber diameter, even when full exchange of troponin for fluorescently labeled troponin is achieved. To characterize the time course of reexchange of fluorescently labeled troponin for unlabelled troponin, fibers that had first incorporated the rhodamine-labeled troponin (Fig. 3 *A*) were incubated in exchange buffer with troponin containing IANBD-labeled troponin I, which is not "visible" in the confocal microscope with the excitation/emission system for rhodamine. Fig. 3 *A*1 (*open circles*) shows that during such a chase experiment, fluorescence decayed to ~20% within 50–80 min. After 15 h, the remaining fluorescence was ~2%, i.e., the exchange was essentially complete. Note that over the same period of incubation in exchange buffer without the IANBD-labeled troponin, only a small decrease in fluorescence occurred (*filled squares*). This indicates that the decay in fluorescence in the chase experiment results from the exchange of Rh-labeled TnI for the "nonvisible" IANBD-labeled TnI and is not due to spontaneous loss of TnI from the fibers. Effects from bleaching during the test scans with the confocal laser scanning microscope were ruled out by comparison with other areas of the fibers that were not scanned before. The time course of this "chase experiment" agrees well with the exchange followed by PAGE analysis (Fig. 2). Note, however, that with cardiac troponin, used for the PAGE analysis, somewhat longer times might be required for complete exchange of the native skeletal troponin.

To determine whether the total intensity reported by imaging the fiber onto the photomultiplier device follows a time course similar to the equilibration followed by confocal microscopy, we fitted our confocal scanner directly to the home-built inverted microscope. Using a 20× water immersion objective (Achroplan 20×/0.50 w; Carl Zeiss, Jena, Germany), we could record confocal images while the fibers were mounted in our mechanical setup. After slight differences over the first few minutes, the time course of total intensity of photomultiplier output and the time course of integrated intensity within profiles across a longitudinal section through the core of the fiber by confocal imaging (cf. Fig. 1) were essentially identical.

Fig. 3 *B* shows the labeling pattern of two fibers that were incubated at two different sarcomere lengths. Both fibers were incubated for 2 h in exchange buffer with 0.3 mg/ml of troponin with Rh-labeled TnI. Note that the labeling of the thin filament is not homogeneous throughout its whole length, but rather appears to be more prominent within the area of overlap between thick and thin filaments. For the procedure for identifying the various elements of the sarcomere, see Fig. 3 *A* (*arrows* indicate the M-line position). The localization of fluorescence to the area of overlap again indicates that fluorescent troponin is not just binding to the actin filament nonspecifically, but that instead true exchange occurs, which is promoted by strongly attached cross-bridges. This is supported by the observation that under relaxing conditions (no attachment of cross-bridge in strong binding states) no substantial exchange could be achieved (data not shown). The apparent preferential exchange of troponin within the overlap region is in contrast to the extraction of troponin C, which appears to occur preferentially in the nonoverlap region (Yates et al., 1993), while the rebinding of troponin C to myofibrils that had been extracted to remove virtually all of the troponin C did occur primarily in the overlap region (Swartz et al., 1997). Thus it appears that strong cross-bridge attachment affects exchange by the various procedures in different ways.

Equatorial x-ray diffraction patterns

Widths and intensities of the two innermost equatorial reflections (1,0 and 1,1 reflections) of x-ray diffraction patterns are sensitive to both disorder in the myofilament lattice and addition of mass to the thin filament (Haselgrove and Huxley, 1973; Yu et al., 1985). Thus recording of equatorial diffraction patterns allowed us to test whether our exchange protocol had affected the submicroscopic structure of skinned fibers, as we had seen before when using the standard protocols for extraction/reconstitution of troponin C (TnC) (e.g., Zot and Potter, 1982; Moss et al., 1985; Babu et al., 1986; our own unpublished results). After incubation for 2 h in exchange buffer with 0.5 mg/ml of Rh-labeled skeletal troponin (Rh-labeled TnI incorporated into troponin), after which time >80% of native troponin is expected to be replaced by externally applied troponin (cf. Fig. 3 *A*1), there was no detectable change in the equatorial diffraction pattern, e.g., in the widths or intensities of the two innermost reflections. In fact, the patterns before and after exchange were indistinguishable (Fig. 4). This indicates that no detectable structural disorder is introduced by the exchange protocol and that no detectable amount of additional mass becomes associated with the thin filaments during the incubation period. That significant incorporation of rhodamine-labeled TnI had been accomplished during these test incubations was verified by confocal microscopy, the reason for using Rh-labeled troponin (Rh-TnI) in this control study.

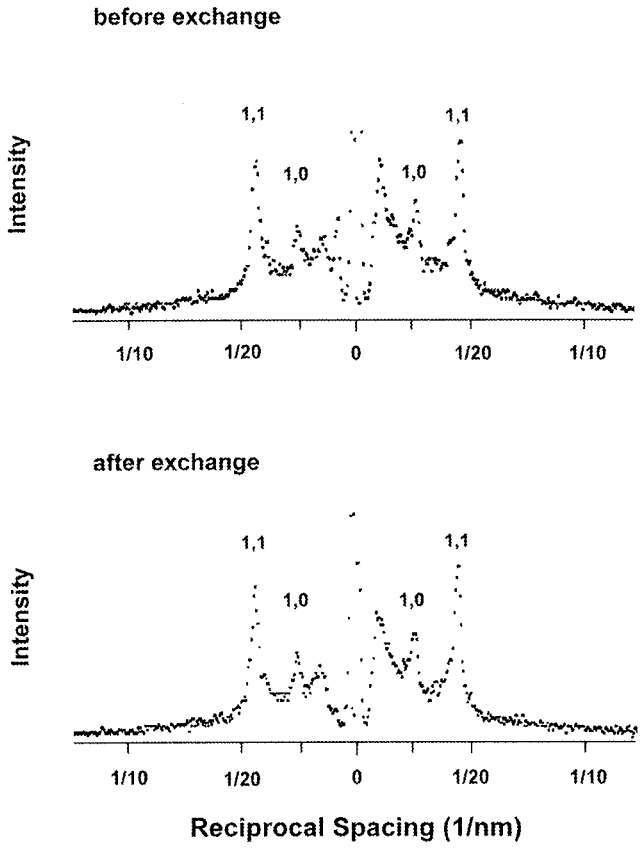

FIGURE 4 Equatorial diffraction patterns recorded before and after 3 h of incubation in exchange buffer with 0.5 mg/ml of rhodamine-labeled troponin (Rh-labeled TnI). Note there is no significant change in the equatorial intensities (I1,0 and I1,1), the width of the reflections, or background scatter; i.e., the two patterns are essentially indistinguishable.

Mechanical properties before and after exchange of native troponin for fluorescently labeled troponin

To test whether the exchange of endogenous troponin for IANBD-labeled troponin changes the normal function of the skinned fibers, we examined active force and the rate constant of force redevelopment at maximum and submaximum Ca^{2+} (Fig. 5). At saturating Ca^{2+} concentrations active tension and the rate constant of force redevelopment were reduced by less than 5% and 10%, respectively, after the exchange procedure (Fig. 5 *a*). The procedure also had little if any effect on force-pCa relations and plots of k_{redev} versus force (Fig. 5, *b* and *c*), suggesting that there was no significant loss of, e.g., troponin C during our exchange protocol.

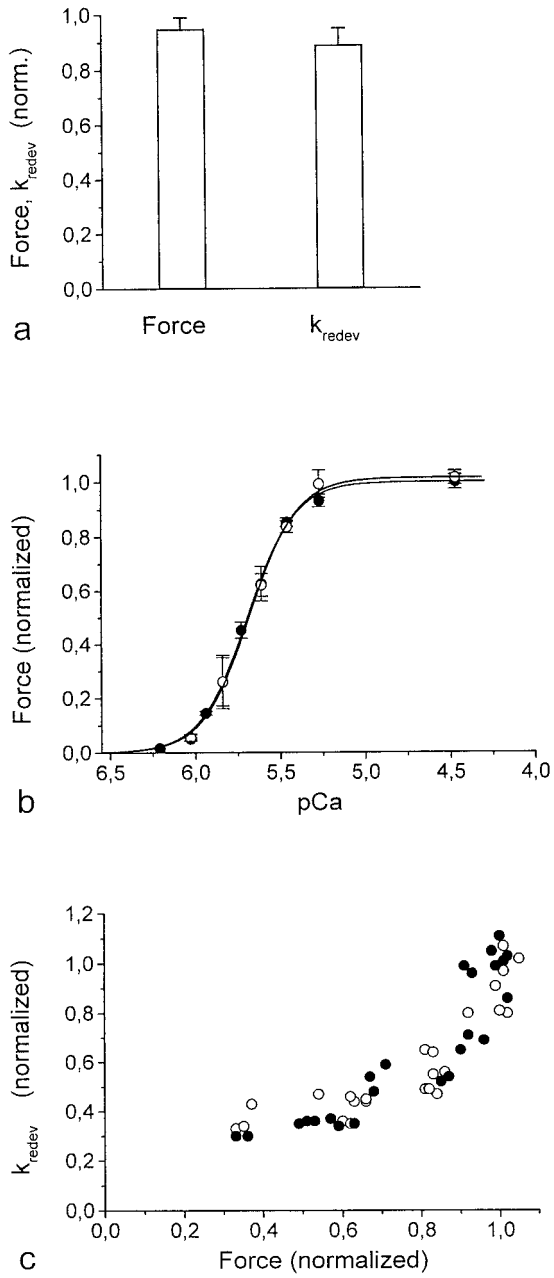

FIGURE 5 Mechanical parameters before and after exchange of endogenous troponin for fluorescently labeled troponin (IANBD-labeled TnI-subunit). (*a*) Isometric force and rate constant of force redevelopment (k_{redev}) at saturating Ca^{2+} concentrations. Data of three fibers, means ± SD. (*b*) Force-pCa relations. ●, Before exchange; ○, after exchange. Solid lines are fits of the sigmoidal function to data points (means ± SD). Note that even at low activation levels there is no significant change in the force-pCa relation, indicating that full regulation is preserved. (*c*) Force versus k_{redev} for different degrees of Ca^{2+} activation. ●, Before exchange; ○, after exchange.

IANBD fluorescence in different states: reconstituted versus native thin filaments

Previous solution studies showed that for reconstituted thin filaments the fluorescence of IANBD-labeled TnI is decreased by Ca^{2+} binding to TnC or by attachment of myosin subfragment 1 in a strong-binding state (Trybus and Taylor, 1980). We therefore compared the behavior of IANBD-labeled TnI in response to Ca^{2+} and attachment of cross-bridges in strong binding states to reconstituted actin in solution with the behavior of IANBD-TnI fluorescence after exchanging the labeled troponin complex into native thin filaments of the skinned fibers.

Solution studies

Fig. 6 is a summary of several solution measurements of fluorescence changes of IANBD-TnI in response to Ca^{2+} and S1 binding in the absence of nucleotide. The fluorescence in the absence of Ca^{2+} and S1 was defined as 1.0, while the lowest value of fluorescence in a particular series, i.e., in the presence of both Ca^{2+} and S1, was defined as zero. Note that in the figure, values of zero were offset by 0.015 for clarity. Measurements were made at both 56 mM and 155 mM ionic strength. The addition of Ca^{2+} alone caused a reduction in fluorescence by, on average, 84% of the maximum change in fluorescence. However, note that in two out of seven trials Ca^{2+} alone also resulted in the maximum possible inhibition of fluorescence. The addition of saturating amounts of S1, in the absence of free Ca^{2+}, reduced the fluorescence by only 52% of the maximum change, i.e., less than that seen with Ca^{2+} alone. The fluorescence was reduced to its minimum value in the presence of both Ca^{2+} and S1. Using the same probe, Trybus and Taylor observed that at 220 mM ionic strength, Ca^{2+} alone caused 80% of the total change seen in the presence of both S1 plus Ca^{2+}, while at 20 mM ionic strength Ca^{2+} alone caused only one-third of the total effect. The results at 220 mM ionic strength are similar to ours in that respect. Trybus and Taylor also found that either Ca^{2+} alone or S1 alone gave ~80% of the total change, whereas, in our hands, S1 alone gives only ~50% of the total change. The difference between our preparation of IANBD-troponin and theirs is that subsequent to labeling we removed tropomyosin from the fluorescently labeled troponin by hydroxyapatite chromatography and reconstituted with unmodified tropomyosin. Normally 10% of the tropomyosin was labeled during reaction of the troponin-tropomyosin complex with IANBD. In heavily labeled preparations, up to ~10% of the total label was on troponin C and a trace amount was on troponin T. The variation in the labeling of TnC may contribute to the differences observed. The labeling of TnC and the trace labeling of TnT are not easily observable, and there is no way of knowing the extent to which this was a factor in other laboratories using this same labeling procedure.

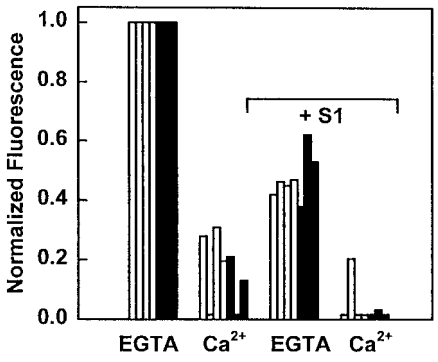

FIGURE 6 IANBD-troponin fluorescence under different conditions in solution. Each bar represents a separate measurement of fluorescence with excitation at 492 nm and emission at 538 nm. Conditions: 3.5 μM actin, 0.6 μM troponin-tropomyosin, and either 0 or 3.5 μM myosin subfragment 1 (S1) at 10°C in a final volume of 2.86 ml of 20 mM imidazole, pH 7.0, 2 mM $MgCl_2$, either 34 mM (□) or 134 mM potassium propionate (■) and either 2 mM EGTA or 2 mM CaEGTA. The troponin was labeled on the TnI subunit with IANBD. The fluorescence was first measured in the absence of Ca^{2+} and then again on the same sample after the addition of $CaCl_2$. For ease of comparison, the data were normalized such that fluorescence in the absence of both Ca^{2+} and S1 was set at 1.0, while the lowest fluorescence value in any single experiment (in the presence of Ca^{2+} and S1) was defined as zero. Fluorescence was normalized so that experiments done with different protein preparations could be compared. The actual decrease in fluorescence was ~16%. Note that fluorescence of the free troponin-tropomyosin complex is only about half the fluorescence of the troponin-tropomyosin complex bound to actin and does not change when the Ca^{2+} concentration is altered. Thus, for 0.1 μM of free troponin-tropomyosin complex, at most ~10% of total fluorescence seen in the absence of Ca^{2+} is Ca^{2+}-insensitive fluorescence originating from the free troponin-tropomyosin complex.

Skinned fiber studies

Effect of weak binding states of the myosin head

The effect of actin interaction of weak binding states of the myosin head on the fluorescence of IANBD-labeled TnI was examined for relaxed fibers (low Ca^{2+}) in the presence of MgATP and in the presence of the nucleotide analog MgATPγS. MgATPγS is a slowly hydrolyzed ATP analog (Goody and Eckstein, 1971) for which the elementary cleavage step is ~500-fold slower than for MgATP (Bagshaw et al., 1973). In fibers, the rate of MgATPγS hydrolysis is not affected by Ca^{2+} (Dantzig et al., 1988; Kraft et al., 1992). We previously showed that cross-bridges with MgATPγS cannot activate the thin filament (Kraft et al., 1992; Resetar and Chalovich, 1995); i.e., cross-bridges with ATPγS in the nucleotide binding site represent weak binding states of the myosin head. As shown in Fig. 7 *b*, at low Ca^{2+} concentration, IANBD fluorescence is essentially the same in the presence of MgATP or MgATPγS. Moreover, when ionic strength is lowered in the presence of MgATP under relaxing conditions, no change in fluorescence is seen (Fig. 7 *c*), despite an increasing number of myosin heads in weak binding states becoming attached to actin (cf. Brenner et al., 1984, 1986).

Effect of Ca^{2+} in the presence of MgATP or MgATPγS. Raising Ca^{2+} to saturating levels (pCa 4.5) results in a quenching of fluorescence by some 50%, not only in the presence of MgATP (Fig. 7), when fibers are generating full isometric tension, but also in the presence of MgATPγS (Fig. 7 *b*), where fibers do not generate any detectable active force (<1–2% of the maximum isometric force that would be our detection limit). This indicates that at saturating Ca^{2+} concentrations maximum inhibition is observed irrespective of the presence (MgATP) or absence (MgATPγS) of cross-bridges in their strong binding states, as has also been described meanwhile for probes on TnC (Martyn et al., 1999).

In solution, the magnitude of the fluorescence change of IANBD-labeled troponin upon activation with both a high Ca^{2+} concentration and S1 binding was previously reported to be relatively insensitive to ionic strength. In contrast, the

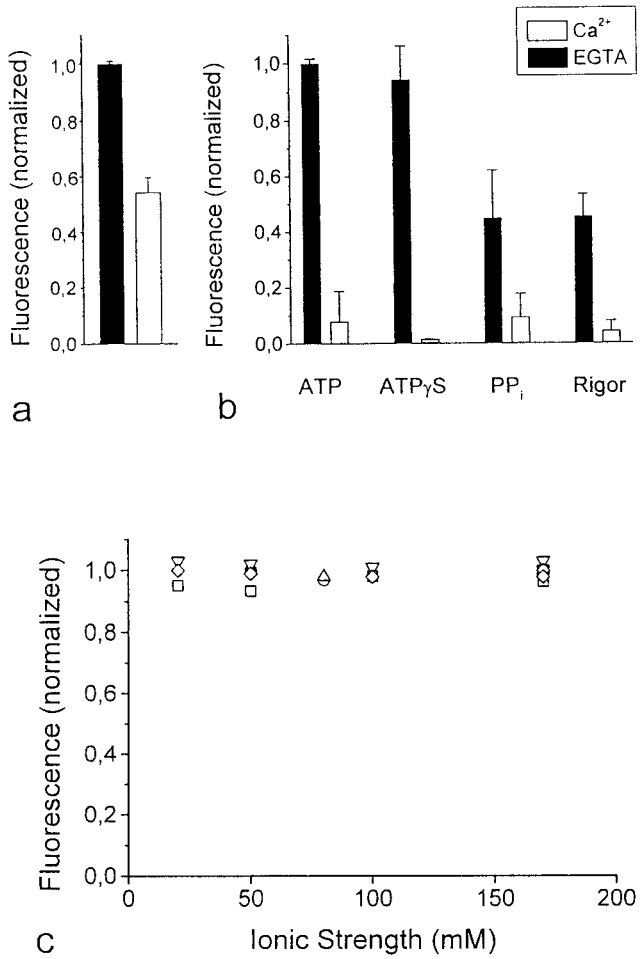

FIGURE 7 IANBD fluorescence observed in skinned fibers under different experimental conditions. (*a*) Fluorescence intensity in the presence of MgATP at low (pCa ~8; ■) and high (pCa 4.5; □) Ca^{2+} concentrations. The data are from six fibers; data were normalized to the average intensity recorded under relaxing conditions (means ± SD). (*b*) Effect of Ca^{2+} at different nucleotide conditions. Here the change in fluorescence with the addition of Ca^{2+} is normalized to the maximum change between highest fluorescence (ATP, no Ca^{2+}) and minimum fluorescence (ATPγS + Ca^{2+}). To show the lowest level, the data for ATPγS + Ca^{2+} are offset by 0.015. The data of six fibers are summarized; data are means ± SD. Note that two extreme levels of fluorescence are observed, one in the absence of Ca^{2+} when cross-bridges are detached or only weakly bound to actin (low Ca^{2+}, with MgATPγS or MgATP). The other level is seen in the presence of saturating Ca^{2+} when cross-bridges are attached to actin in either their strong binding states ($MgPP_i$, rigor) or their weak binding states (MgATPγS), or both (MgATP). An intermediate level is observed when cross-bridges in their strong binding states attach to actin in the absence of Ca^{2+} ($MgPP_i$, rigor). (*c*) Effect of ionic strength under relaxing conditions. Each symbol represents an individual fiber (total of five fibers).

fraction of the total fluorescence change due solely to the addition of Ca^{2+} increased with increasing ionic strength (Trybus and Taylor, 1980). To determine the ionic strength effect on the fluorescence change upon the addition of Ca^{2+} in fibers, it was necessary to stretch the fibers out of filament overlap to avoid attachment of cross-bridges in strong binding states in the presence of MgATP when Ca^{2+} was raised. Simple replacement of ATP with ATPγS could have been used as an alternative method to prevent active cross-bridge turnover with the attachment of cross-bridges in their strong binding states at high Ca^{2+} concentrations. However, the 10 mM MgATPγS needed for full nucleotide saturation at high Ca^{2+} concentrations (Kraft et al., 1992) would have precluded extending the ionic strength dependence to values below 63 mM.

Table 1 shows that there is no statistically relevant ($p > 0.05$) effect of ionic strength on the inhibition of fluorescence by Ca^{2+} alone. In the fiber, as also seen for two out of seven trials in solution, Ca^{2+} alone gives the maximum fluorescence change with little additional effect by attachment of myosin heads in their strong binding states (Fig. 7 *b*, ATPγS + Ca^{2+} versus ATP + Ca^{2+}, or $MgPP_i$ and rigor at high Ca^{2+}; see next paragraph). Furthermore, in our solution studies, unlike the earlier report of Trybus and Taylor (1980), there is little effect of ionic strength on the maximum change in fluorescence.

Fluorescence in rigor and in the presence of 4 mM $MgPP_i$. At saturating Ca^{2+} concentrations, the fluorescence in rigor and in the presence of 4 mM $MgPP_i$ is reduced to essentially the same level as seen at saturating Ca^{2+} concentrations in the presence of MgATP or MgATPγS. However, in the absence of Ca^{2+}, the fluorescence of fibers in rigor or in the presence of 4mM $MgPP_i$ is intermediate between that of relaxed fibers and the fluorescence at saturating Ca^{2+} concentrations (Fig. 7 *b*).

It is conceivable, however, that the inhibition of fluorescence with the attachment of cross-bridges in their strong binding states ($MgPP_i$, rigor) is an indirect effect resulting from an increased Ca^{2+} affinity of TnC. In that case the effect of strong cross-bridge attachment to actin when no Ca^{2+} is added to the solutions results from increased binding of contaminant Ca^{2+} to the regulatory proteins. To clarify this question we studied the effect of Ca^{2+} titration on the IANBD-TnI fluorescence in rigor, where all cross-bridges occupy the strong-binding-type rigor state. If attachment of cross-bridges in their strong binding states has a direct inhibitory effect, then as free Ca^{2+} is lowered, fluorescence should not reach the relaxed level, but rather a Ca^{2+}-independent level between the fully activated (high Ca^{2+}) level and the fully relaxed (low Ca^{2+}) level in the presence of ATP or ATPγS values. As shown in Fig. 8, as Ca^{2+} concentration is lowered, IANBD fluorescence under rigor conditions approaches a Ca^{2+} independent level that is significantly lower than the relaxed level (*filled triangle*) and about half way between relaxed and fully Ca^{2+}-saturated levels. Note that for fibers out of overlap (i.e., without cross-bridge attachment), fluorescence is independent of nucleotide (cf. *open triangle* versus *open circle* at low Ca^{2+} concentration), and the fluorescence-pCa relation is shifted by about an order of magnitude to higher Ca^{2+} concentrations. This observation suggests that actin interaction of cross-bridges in their strong binding states both inhibits IANBD-TnI fluorescence directly and shifts the fluorescence-pCa relation to lower Ca^{2+} concentrations, presumably by increasing the Ca^{2+} affinity of TnC. Other factors

TABLE 1 Inhibition of IANBD fluorescence by Ca^{2+} for fibers stretched beyond filament overlap

Ionic strength	20 mM	50 mM	100 mM	170 mM
Inhibition of fluorescence	0.78 ± 0.12	0.84 ± 0.05	1.00 ± 0.05	0.81 ± 0.03

After exchange of native troponin for IANBD-labeled troponin at sarcomere lengths between 2.3 and 2.4 μm, fibers were stretched to sarcomere lengths where no overlap between actin and myosin filaments can occur (sarcomere length $\geq$ 4.2 μm). The observed extent of reduction in fluorescence at saturating Ca^{2+} concentrations is normalized to the maximum reduction that was seen at 100 mM ionic strength. Numbers are means ± SD, $n = 3$. SD for 100 mM results from taking the average of all measurements at 100 mM ionic strength as 1.0 and calculating the SD from the scatter of the individual measurements around the mean. Note that the effects of Ca^{2+} on the magnitude of the fluorescence changes are not statistically different ($p > 0.05$) at the different ionic strengths examined. At 100 mM ionic strength the decrease in fluorescence with the addition of Ca^{2+} was 20 ± 2% of the fluorescence observed at low Ca.

associated with stretching fibers out of overlap, like changes in lattice spacing or some loss of TnC when exchanging at long sarcomere lengths, however, might also contribute to the shift in the fluorescence-pCa relation.

Nevertheless, these data indicate that the IANBD probe on troponin I, in principle, is sensitive to activation of thin filaments by calcium and by actin attachment of cross-bridges in their strong binding states, although the magnitude of the change is different in the two cases (Fig. 7). Note, however, that attachment of cross-bridges in their strong binding states in addition to saturating Ca^{2+} concentration does not further reduce fluorescence (Fig. 7 *b*), in contrast to the biochemical studies in solution (Trybus and Taylor, 1980; Fig. 6, but also note that two trials in our solution studies also gave full inhibition by calcium alone).

IANBD fluorescence at different degrees of Ca^{2+} activation. Next we characterized the relation between IANBD-TnI fluorescence and the level of Ca^{2+} activation in the presence of ATP, i.e., with active cross-bridge turnover (Fig. 9). To characterize the contribution from force-generating cross-bridges we first recorded IANBD fluorescence in full overlap (*open squares*). Then fibers were stretched out of filament overlap and fluorescence was recorded again at different degrees of Ca^{2+} activation (*open circles*). The lower fluorescence intensities at long sarcomere length are due to fewer sarcomeres (i.e., fewer fluorophores) in the field of view. In full overlap, the relation between IANBD fluorescence and pCa appears to be shifted toward lower Ca^{2+} concentrations compared to the out-of-overlap condition. This becomes more evident in Fig. 9 *b*, where fluorescence data for both long and short sarcomere lengths were normalized to their respective values under relaxing conditions. The shift to lower Ca^{2+} concentrations and the somewhat steeper pCa-relation suggests some contribution to the change in fluorescence by force-generating, strong binding cross-bridges, at least at nonsaturating Ca^{2+} concentrations. From all fibers studied, the inhibition of fluorescence upon maximum Ca^{2+} activation (pCa 4.5) was 46 ± 10% ($n = 6$) in full overlap versus 44 ± 6% ($n = 4$) out of overlap. This suggests that at saturating Ca^{2+} concentrations actin attachment of endogenous cross-bridges that occupy strong binding states has little additional effect on the reduction of fluorescence, which is consistent with the rather similar fluorescence observed under any nucleotide condition at high Ca^{2+} concentrations (cf. Fig. 7 *b*). A similar observation with probes on TnC has recently been described by Martyn et al. (1999).

DISCUSSION

The present study shows that whole troponin can be exchanged into skinned fibers of rabbit psoas muscle without detectable alteration of their mechanical and structural properties. This procedure allows the incorporation of alternative types of troponin, such as cardiac troponin, as well as modified skeletal troponins such as the fluorescently labeled troponin used here. Gel electrophoresis was used to verify that troponin I and troponin T are exchanged. Exchange instead of unspecific binding is also demonstrated by the change in the force-pCa relationship that occurred when the native skeletal muscle troponin was exchanged for cardiac troponin. Confocal microscopy also verified exchange by showing that the fluorescent troponin is preferentially located in the region of filament overlap.

The exchange procedure apparently does not degrade structural or functional properties of the fibers. 1) The equatorial reflections of x-ray diffraction patterns are unchanged after incorporation of externally applied troponin. Changes in intensities or widths of reflections would have occurred if there had been significant structural changes. 2) Isometric force, force redevelopment, and force-pCa relations are virtually unchanged after the exchange procedure. This is a significant improvement over other methods that we have tried that are based on the extraction of TnC followed by reconstitution (Zot and Potter, 1982; Moss et al., 1985; Babu et al., 1986). These previously developed methods did not give as great an extent of exchange; the force is not fully restored after reconstitution, especially when the extent of exchange is maximized. In addition, equatorial diffraction patterns suggested significant structural disorder (own unpublished observations). One method that we have not tested involves the extraction of TnI and TnC with an excess of TnT (Nakayama et al., 1990; Shiraishi et al., 1992; J. Potter, personal communication).

The structural and functional preservation during our exchange process may be due to the near-physiological conditions for the exchange and the avoidance of unpaired protein interactions. The exchange process used here requires reversible dissociation of regulatory proteins or parts of the native regulatory protein complexes. Under normal conditions the detached proteins or detached parts of the native complexes rebind rapidly to unoccupied binding sites, and diffusion of these proteins out of the highly condensed structure of skinned fibers cannot effectively occur unless the binding affinity is drastically lowered by

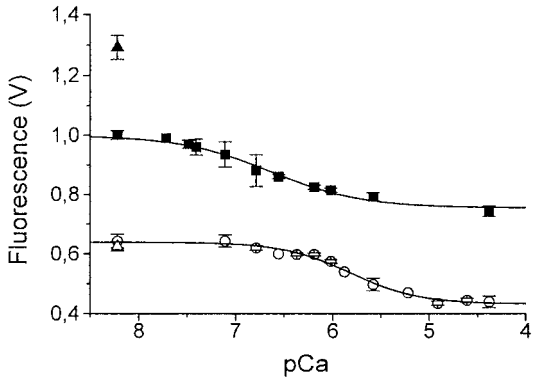

FIGURE 8 Effect of different Ca^{2+} concentrations under rigor conditions in full filament overlap (SL = 2.4 μm; ■) and when fibers were stretched beyond filament overlap (SL ≥ 4.2 μm; ○). ▲, △, Fluorescence of relaxed fiber (MgATP, pCa 8.2) at short and long sarcomere lengths, respectively. All symbols are means ± SD. Note that at the short sarcomere length (full filament overlap) fluorescence at low Ca^{2+} concentrations reaches a plateau that is about half-way between relaxing and saturating Ca^{2+} concentrations; i.e., fluorescence under rigor conditions does not reach the relaxed level when Ca^{2+} is reduced (Fig. 7 *b*). At long sarcomere lengths, fluorescence at low Ca^{2+} is independent on nucleotide (no cross-bridge interaction with actin filaments, i.e., no interference of attached cross-bridges with regulatory proteins). The lower fluorescence intensity at the long sarcomere length results from the much reduced number of fluorophores in the field of view.

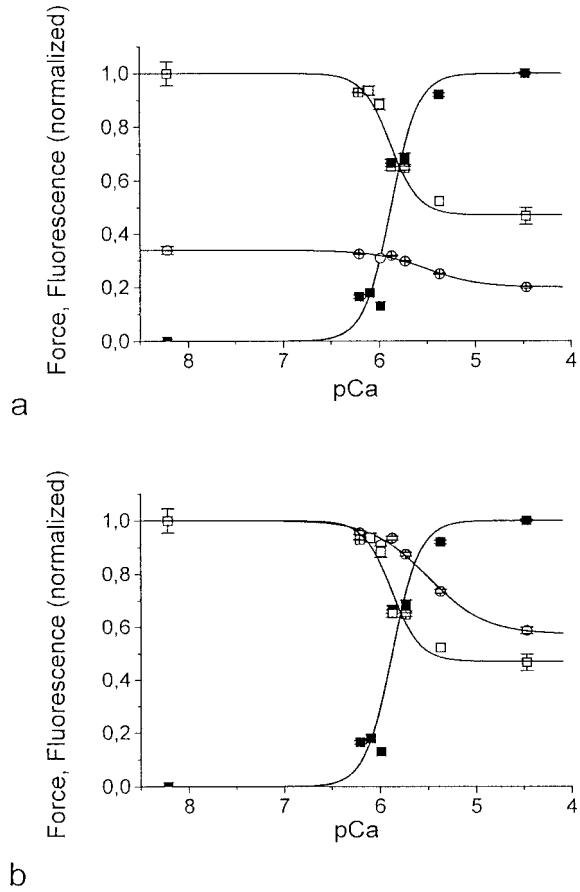

FIGURE 9 (*a*) IANBD fluorescence at different Ca^{2+} concentrations in full filament overlap (□; sarcomere length 2.4 μm) and out of overlap (○; sarcomere length ≥4.2 μm). ■, Active force versus pCa (in full filament overlap). Fluorescence data are normalized to fluorescence observed in overlap in the absence of Ca^{2+}. To illustrate the somewhat steeper profile of fluorescence versus pCa in full filament overlap, in *b* fluorescence data for both sarcomere lengths are normalized to their respective maximum values under relaxing Ca^{2+} concentrations. Data are from one representative fiber.

imposing highly nonphysiological conditions. In the case of an exchange reaction, however, sites that become transiently unoccupied under near-physiological conditions are rapidly filled by competition with externally applied molecules (cf. Kraft et al., 1995b).

Because exchange occurs preferentially in the region of overlap, it is necessary to exert care when interpreting experiments specifically when the sarcomere length is changing. For instance, if the exchange is not uniform along the length of the thin filament, then as the fiber shortens, some cross-bridges may move into an unlabeled region of the actin filament. In this case, cross-bridges that contribute to the mechanical properties of the fiber will be invisible in terms of fluorescence changes. This problem can be avoided by measuring the sarcomere length during labeling and during experimentation. It is not likely that the nonexchanged parts of the thin filaments in the I-band will complicate the fiber behavior as a result of cooperative effects along the thin filaments. In fibers and myofibrils these cooperative effects appear to be limited to rather short distances (cf. Swartz et al., 1990; Kraft et al., 1995b).

IANBD-fluorescence is sensitive to both actin interaction of cross-bridges in strong binding states and Ca^{2+} binding to troponin C

Our data show that in fibers, as in solution, both an increase in Ca^{2+} and actin interaction of cross-bridges in their strong binding states reduce the fluorescence of IANBD-labeled troponin I. In fibers, Ca^{2+} alone (no attachment of cross-bridges in strong binding states, e.g., in the presence of MgATPγS) results in maximum inhibition of fluorescence, while attachment of cross-bridges in strong binding states alone (rigor cross-bridges and cross-bridges with $MgPP_i$ at relaxing Ca^{2+} concentration) results in only half-maximum fluorescence change. Studying the effect of different Ca^{2+} concentrations on IANBD-TnI fluorescence in the presence of cross-bridges in strong binding states (e.g., in rigor) suggests that the inhibition of fluorescence in this condition results directly from actin interaction of cross-bridges in strong binding states and not just from increased Ca^{2+} affinity of troponin C (cf. Fig. 8).

There are two differences between the changes seen in fibers with changes reported earlier with IANBD-labeled TnI in solution (Trybus and Taylor, 1980). First, in fibers maximum fluorescence change was already seen upon the addition of calcium alone, whereas in solution, ~80% of fluorescence change occurred with calcium alone and additional strong cross-bridge attachment was required for max-

imum fluorescence change (cf. Fig. 6; Rosenfeld and Taylor, 1985). Second, in solution, saturating calcium alone or attachment of strong binding cross-bridges at low Ca^{2+} yields similar degrees of fluorescence change, while in fibers attachment of strong binding cross-bridges at low calcium gave only 50% of the maximum change seen at saturating calcium.

The differences in behavior may have several potential causes. One variable is the degree of labeling of components other than troponin I during the labeling procedure. This occurs because the labeling is done with the intact troponin-tropomyosin complex (cf. Trybus and Taylor, 1980). We observed that a small but significant (<10%) fraction of the tropomyosin and troponin C could be labeled during this procedure. We separated the troponin from the tropomyosin and reconstituted the regulated thin filaments for solution studies with unlabeled tropomyosin. Therefore, in both our fiber and solution experiments no fluorescence changes could originate from fluorescently labeled tropomyosin. We note that in our own solution studies done with the same proteins used for the fiber studies we observed a better agreement between solution and fiber studies. It is particularly noteworthy that at low calcium concentrations the addition of myosin subfragment 1 (S-1) without nucleotide (rigor, strong binding state) gave only a 50% fluorescence change in both solution and fibers. Fibers always give a greater percentage inhibition of fluorescence with calcium alone and even in two of seven of our solution experiments we observed full inhibition of fluorescence with calcium alone. At subsaturating calcium concentrations, however, we do observe a further fluorescence decrease upon binding to strong binding cross-bridges in the fibers. Additional factors that affect calcium affinity or protein fluorescence may be keys to qualitative differences between solution and fiber studies. For instance, sarcomeric proteins not present in solution studies may alter the calcium sensitivity in addition to effects on the magnitude of the fluorescence changes (e.g., 50% maximum change in fibers versus <20% in our solution studies). It is also possible that in solution reconstitution either is incomplete or might involve (partially) denatured troponin molecules such that some regulated units of the actin filaments do not respond to Ca^{2+} and only become activated by strong (e.g., rigor-like) cross-bridge attachment.

The lack of further fluorescence change upon strong cross-bridge attachment in the presence of saturating calcium does not necessarily mean that Ca^{2+} is sufficient to achieve full activation of muscle fibers. It is possible that further changes occur in the regulatory proteins upon strong cross-bridge attachment that do not result in further changes in TnI fluorescence. However, other work from our laboratory does suggest that Ca^{2+} alone is sufficient to achieve full or nearly full activation. For instance, diffusing *N*-ethyl maleimide modified myosin subfragment 1 into skinned fibers did not result in any further increase in the rate constant of force redevelopment than that seen at saturating Ca^{2+} concentrations (Kraft et al., 1993). In addition, no change in cross-bridge cycling kinetics (e.g., rate constant of force redevelopment, ratio of ATPase over tension) was observed when force generation was inhibited by the addition of caldesmon (Kraft et al., 1995a). A complete answer to this question, however, will require additional experiments with fluorescent probes on other sites of the regulatory system.

Fluorescence of IANBD-labeled TnI is not sensitive to actin attachment of cross-bridges in their weak binding states

From our previous work (e.g., Brenner et al., 1984, 1986, 1991; Kraft et al., 1995a) it is expected that under the conditions of Fig. 7 *c* (MgATP at low Ca^{2+}, MgATPγS at low or high Ca^{2+}), i.e., when cross-bridges are in their weak binding states, up to some 80% of all cross-bridges should become attached to actin. Yet attachment of cross-bridges in these weak binding states did not change the IANBD fluorescence. We have made similar observations with weak binding cross-bridge states formed in the presence of GTP and AMPPNP (Heizmann et al., 1997). This is consistent with the concept that attachment of cross-bridges in weak binding states has little or no effect on the state of activation of the regulated thin filament (Chalovich et al., 1983, Brenner, 1987, 1990). Note that Morano and Rüegg (1991) did observe a change in TnC-DANZ fluorescence with the attachment of cross-bridges in their weak-binding states. This observation will be tested using the exchange procedure described in this work. The inability of weak binding cross-bridges to activate the actin filament is independent of experimental conditions (Chalovich et al., 1983). Therefore the fluorescence of IANBD-labeled TnI is a useful probe for distinguishing weak and strong binding states of the myosin at subsaturating free Ca^{2+}, where sizable fluorescence changes may occur.

There has been confusion over the terms "weak binding" and "strong binding," because the affinities of the various myosin states are dependent on conditions such as ionic strength. On the basis of the arguments already presented, we propose to call the classical weak binding states of the myosin head "nonactivating" states and the classical strong binding states "activating." Whether a myosin head is in one of the activating or nonactivating states depends solely on the nucleotide bound to the myosin head and is a property of the myosin head that is independent of whether the myosin head is dissociated from actin or bound to activated or inactive forms of the actin filament.

Actin interaction of non activating ("weak binding") versus activating ("strong binding") states of the myosin head

Although the weak binding states of myosin do not activate actin filaments (as the name "nonactivating" implies), they are responsive to changes in Ca^{2+}. For example, the com-

plex of myosin head and ATPγS is a nonactivating state with only a small Ca^{2+} dependence on the affinity for regulated actin. Yet Ca^{2+} reduces the rate constants for attachment and detachment of myosin-ATPγS to and from regulated actin by ~50- and ~100-fold, respectively (Kraft et al., 1992). That same study showed that the affinity of the myosin head for ATPγS is reduced by up to 1000-fold when the myosin-ATPγS complex binds to regulated actin at high Ca^{2+}-concentrations. Note that the large increase in actin affinity as Ca^{2+} concentration is raised, reported by others (Dantzig et al., 1988), apparently was due to much reduced ATPγS saturation, i.e., to a large fraction of nucleotide-free cross-bridges at high Ca^{2+} concentrations (cf. Kraft et al., 1992).

These findings suggested that even the nonactivating (weak binding) states of myosin change their mode of interaction with regulated actin when the tropomyosin-controlled sites on actin become accessible at high Ca^{2+}, and the following picture appears to emerge: in the absence of Ca^{2+}, myosin heads in both the nonactivating (weak binding) and activating (strong binding) states bind only to sites of electrostatic interaction. An increase in free Ca^{2+} changes the average position of tropomyosin, exposing additional sites for the interaction of myosin heads (Lehman et al., 1994, 1995). As shown by the much slower actin binding kinetics and much reduced nucleotide affinity at high Ca^{2+} in the presence of ATPγS, not only the activating (strong binding) but also the nonactivating (weak binding) states of the myosin head interact with these additional sites, but in very different ways, e.g., resulting in different effects of activation on affinities for nucleotide as well as for actin. It appears that the presence of a γ-phosphate or γ-phosphate analog dictates this different behavior, because other nucleotides or nucleotide analogs with an uncleaved (or only slowly cleaved) γ-phosphate, e.g., MgAMPPNP or MgGTP, show effects similar to those found with MgATPγS (Frisbie et al., 1997; Heizmann et al., 1997).

Geeves and co-workers have proposed, on the basis of their studies, that nonactivating and activating states of myosin pass through identical intermediates when interacting with regulated actin (Geeves et al., 1984; McKillop and Geeves, 1993). Such a concept becomes difficult to justify when one now considers that Ca^{2+} affects both activating and nonactivating myosin states, but in very different ways. We therefore favor the concept that binding of nonactivating and activating states of myosin to regulated actin occurs through at least some different intermediates. Such distinctly different intermediates are consistent with current structural data, which show that some structural changes in the nucleotide binding pocket and in the catalytic domain (specifically the position of loop2) are dependent on the γ-phosphate moiety (Rayment et al., 1993; Fisher et al., 1995; Smith and Rayment, 1995, 1996; Dominguez et al., 1998). Thus, also in structural terms, an activating (strong binding) state (no γ-phosphate, no γ-phosphate analog) may not even transiently pass through an intermediate that is equivalent to that with destabilized actin interaction (low actin affinity) and destabilized nucleotide binding (very low nucleotide affinity) that is seen when nonactivating myosin states interact with both uncontrolled (electrostatic) and tropomyosin-controlled binding sites on the Ca^{2+}-activated thin filament.

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