Detection of Fluorescently Labeled Actin-Bound Cross-Bridges in Actively Contracting Myofibrils

Wendy C. Cooper, Lynn R. Chrin, and Christopher L. Berger
Department of Molecular Physiology and Biophysics, University of Vermont College of Medicine, Burlington, Vermont 05405-0068 USA

ABSTRACT Myosin subfragment 1 (S1) can be specifically modified at Lys-553 with the fluorescent probe FHS (6-[fluorescein-5(and 6)-carboxamido]hexanoic acid succinimidyl ester) (Bertrand, R., J. Derancourt, and R. Kassab. 1995. *Biochemistry.* 34:9500–9507), and solvent quenching of FHS-S1 with iodide has been shown to be sensitive to actin binding at low ionic strength (MacLean, Chrin, and Berger, 2000. *Biophys. J.* 000–000). In order to extend these results and examine the fraction of actin-bound myosin heads within the myofilament lattice during calcium activation, we have modified skeletal muscle myofibrils, mildly cross-linked with EDC (1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide) to prevent shortening, with FHS. The myosin heavy chain appears to be the predominant site of labeling, and the iodide quenching patterns are consistent with those obtained for myosin S1 in solution, suggesting that Lys-553 is indeed the primary site of FHS incorporation in skeletal muscle myofibrils. The iodide quenching results from calcium-activated FHS-myofibrils indicate that during isometric contraction 29% of the myosin heads are strongly bound to actin within the myofilament lattice at low ionic strength. These results suggest that myosin can be specifically modified with FHS in more complex and physiologically relevant preparations, allowing the real time examination of cross-bridge interactions with actin in in vitro motility assays and during isometric and isotonic contractions within single muscle fibers.

INTRODUCTION

Actin and myosin interact in an ATP-dependent manner to produce force and motion during muscle contraction. The cyclic nature of this interaction requires that myosin change its affinity for actin by several orders of magnitude at different stages of its ATPase cycle. In the ATP and ADP · P_i (i.e., the products of hydrolysis) states of the ATPase cycle myosin binds weakly to actin, with an association constant of $\approx 10^3\ M^{-1}$ (Stein et al., 1979). Upon the release of phosphate myosin undergoes a conformational change, greatly increasing its affinity for actin and proceeding through its powerstroke to generate myofilament sliding and/or force generation. In the post-powerstroke states (ADP and rigor (i.e., nucleotide-free)) myosin binds strongly to actin, with association constants in the range of 10^6-$10^7\ M^{-1}$ (Greene and Eisenberg, 1980). While the interaction between actin and myosin described above is well understood from solution studies of purified proteins, the interaction between actin and myosin within the myofilament lattice of muscle fibers and myofibrils is not as well characterized.

Mechanical stiffness measurements have been traditionally used to examine the binding of myosin cross-bridges to actin in intact and skinned muscle fibers. Goldman and Simmons (1977) originally reported that ~80% of the myosin cross-bridges are strongly bound to actin in isometrically contracting muscle fibers. However, other studies have indicated that the fraction of actin-bound cross-bridges in isometrically contracting muscle fibers may actually be much lower than 80%. Cooke et al. (1982) demonstrated using EPR spectroscopy that only 20% of the myosin heads adopt the rigor-like orientation expected for strongly bound cross-bridges in spin-labeled, isometrically contracting, skinned muscle fibers. Consistent with this result, proteolytic digestion experiments on isometrically contracting, cross-linked myofibrils have suggested that only 25% of the myosin cross-bridges are bound to actin at physiological ionic strength (Duong and Reisler, 1989; Berger and Thomas, 1993). The discrepancy in the fraction of actin-bound myosin heads measured in actively contracting muscle fibers and myofibrils may be due, in part, to the underlying assumption in the stiffness measurements that most of the sarcomeric compliance resides within the cross-bridge itself. This assumption has recently been challenged by results from both mechanical (Higuchi et al., 1995) and high-resolution x-ray diffraction (Huxley et al., 1994; Wakabayashi et al., 1994) studies on actively contracting muscle fibers, indicating that less than half of the sarcomere's compliance actually resides within the cross-bridges. More recent mechanical stiffness measurements have attempted to account for the existence of these non-cross-bridge compliances. For example, Linari et al. (1998) have reduced the fraction of actin-bound cross-bridges in isometrically contracting muscle fibers to 43%, but this is still significantly higher than the values of 20–25% reported using other techniques.

Accurate determination of the fraction of actin-bound cross-bridges during unloaded myofilament sliding is also critical to understanding chemomechanical coupling in muscle. Conventional models of muscle contraction assume

Received for publication 30 June 1999 and in final form 14 December 1999.

Address reprint requests to Dr. Christopher L. Berger, Dept. of Molecular Physiology and Biophysics, University of Vermont College of Medicine, Burlington, VT 05405-0068. Tel.: 802-656-0832; Fax: 802-656-0747; E-mail: berger@salus.med.uvm.edu.

0006-3495/00/03/1449/09 $2.00

a tight coupling between the biochemical and mechanical cycles of acto-myosin, with the hydrolysis of one molecule of ATP driving a single active interaction between actin and myosin. However, recent estimates of the chemomechanical coupling ratio from in vitro motility assays have ranged from the conventional 1:1 to a $>$ 5:1 correspondence between the number of productive acto-myosin interactions and the amount of ATP hydrolyzed per myosin molecule. Much of the controversy surrounding this issue arises from estimates of the acto-myosin duty cycle (i.e., the fraction of the ATPase cycle time in which myosin interacts strongly with actin), which have ranged from very low values of ~5% (Uyeda et al., 1990; Harris and Warshaw, 1993) to very high values up to 80% (Harada et al., 1990). Thus, experiments that can more directly measure the acto-myosin interaction under both isometric and isotonic conditions within the myofilament lattice are clearly needed to resolve these important issues regarding the molecular mechanism of muscle contraction.

Lys-553, located in the middle of the second of two helices in one of the putative actin-binding interface regions of myosin involving a helix-loop-helix motif in the lower 50 kD subdomain (Rayment et al., 1993; Milligan, 1996; Mendelson and Morris, 1997), has been specifically modified in myosin subfragment 1 (S1) by the fluorescent probe FHS (6-[fluorescein-5(and 6)-carboxamido]hexanoic acid succinimidyl ester) (Bertrand et al., 1995). In addition, we show in the accompanying paper (MacLean et al., 2000) that iodide quenching of the FHS probe in S1 is sensitive to the strong binding of myosin to actin at low ionic strength. Thus, solvent quenching of the FHS probe by iodide may be a useful signal of cross-bridge binding in the intact myofilament lattice (e.g., muscle myofibrils or skinned fibers), provided that myosin can be specifically labeled at Lys-553 in these more complex preparations as it is in solution. In the current study we have modified skeletal muscle myofibrils with FHS and shown that the myosin heavy chain is the predominant site of labeling. Furthermore, the iodide quenching patterns are consistent with those obtained for myosin S1 in solution (MacLean et al., 2000), suggesting that Lys-553 is indeed the primary site of FHS incorporation. Finally, we have demonstrated that iodide quenching of FHS is capable of detecting actin-bound cross-bridges within the myofilament lattice at low ionic strength.

METHODS

Chemicals and solutions

FHS was purchased from Molecular Probes (Eugene, OR). 4-Hydroxy-TEMPO (4-hydroxy-2, 2, 6, 6-tetramethylpiperidinyloxy) and potassium iodide were purchased from Aldrich (Milwaukee, WI). ATP (adenosine 5′-triphosphate), βME (β-mercaptoethanol), EDC (1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide), PPi (tetrapotassium pyrophosphate), trypsin, and all other reagents, at least analytical grade and of the highest purity possible, were purchased from Sigma (St. Louis, MO). Rigor buffer and all other experimental solutions contained 10 mM MOPS, 10 mM KCl, 2 mM $MgCl_2$, 1 mM EGTA, pH 7.0 at 25°C. Relaxing solutions contained an additional 5 mM MgATP and 24 mM EGTA, and activating solutions contained an additional 5 mM MgATP and 2 mM $CaCl_2$.

Myofibril isolation and labeling

Myofibrils were prepared from fresh rabbit leg muscle as previously described (Knight and Trinick, 1982). Myosin concentration in labeled and unlabeled myofibril solutions was determined using a Coomassie protein assay (Pierce, Rockford, IL), assuming half the myofibril protein mass was myosin. Fresh myofibrils were labeled with FHS by a modification of the method for labeling myosin subfragment 1 (S1) of Bertrand et al. (1995). This procedure predominantly modifies Lys-553 of the myosin heavy chain with FHS. Myofibrils were washed with labeling solution (5 mM ATP, 8 mM $MgCl_2$, 25 mM EGTA, 20 mM MOPS, 80 mM KCl, pH 7.0 at 4°C), and then treated with 0.1% Triton in labeling solution for 10 min on ice followed by another wash in labeling buffer. The detergent-extracted myofibrils were then reacted with FHS at a molar ratio of 3:1 (FHS/myosin head) in labeling buffer for 1 h on ice. The labeling reaction was stopped with the addition of 10 mM glycine ethyl ester and incubated on ice for 10 min. The myofibrils were then washed with labeling buffer and incubated on ice for 10 min in labeling buffer plus 20 mM TAPS (pH 8.5) to remove any FHS that was covalently bound to sites other than Lys-553, including other amino acids with primary amines or sulfhydryl groups. The myofibrils were then given a final 2–3 washes in labeling buffer. Myofibrils were stored at −20°C in a solution of 5 mM MOPS, 0.5 mM EGTA, 4 mM $MgCl_2$, and 50% glycerol at pH 7.0. Before use the stored myofibrils were first washed several times in S1 buffer (10 mM MOPS, 1 mM EGTA, 8 mM $MgCl_2$, pH 7.2 at 4°C).

To ensure that the 50 kD tryptic fragment of myosin containing Lys-553 was the primary site of FHS incorporation within the myofibrils, limited tryptic digestion was carried out on the FHS-labeled myofibrils. Myofibrils were concentrated to ~15 mg/ml and resuspended in a small volume of S1 buffer plus 5 mM MgPPi. Trypsin was added at a ratio of 1 mg to 500 mg myofibrils. At various time points up to a total of 15 min, 20 μl of reaction mixture was added to a solution of 75 μl sample buffer (63 mM Tris-HCl, pH 6.8, 10.5% glycerol, 0.02% SDS), 2 μl 14.2 M β-mercaptoethanol, and 5 μl 0.25 M Tris-HCl plus 2 M Urea, pH 8.0, and then boiled for 4 min in preparation for SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Digested and non-digested samples of FHS-myofibrils were run on either a 7.5% or 4–20% gradient SDS-polyacrylamide gel by the method of Laemmli (1970). Fluorescent labeling of myofibrillar proteins was visualized on a UV light box, and the gel was then Coomassie stained for direct visualization. Photographs of the fluorescently imaged gels were digitized and quantified on a Discovery Series desktop scanner interfaced with Quantity One analysis software (v. 2.4, PDI, Huntington Station, NY) (Berger et al., 1996).

Actively contracting myofibrils were first mildly cross-linked with EDC under rigor conditions to prevent shortening as described previously (Duong and Reisler, 1989; Berger and Thomas, 1993). Briefly, myofibrils were adjusted to 1 mg/ml in 0.1 M NaCl/0.1 M MES, pH 6.5 (4°C), and EDC was added to a final concentration of 10 mM. The myofibrils were constantly stirred on ice for 30 min during the cross-linking reaction. The reaction was stopped with the addition of 0.3 M βME, and the myofibrils were washed several times with S1 buffer.

ATPase assays

Myofibrillar ATPase assays were performed under relaxing or activating conditions as described previously (Ludescher and Thomas, 1988). The myofibrillar suspension (0.2–0.5 mg/ml) was continuously stirred in a 1-ml incubation vial containing the appropriate buffer in a thermostatically controlled water bath at 25°C. The reaction was initiated by the addition of

5 mM ATP, and small (50 μl) aliquots were removed at 1-min intervals and assayed colorimetrically for inorganic phosphate by the method of Lanzetta et al. (1979). The steady-state rate of phosphate production per myosin head per second (assuming 50% of the myofibrillar mass is myosin) was determined by a linear regression analysis of the data from the linear phase of the reaction during the first 10 min of the incubation.

Fluorescence spectroscopy

Fluorescence emission spectra were collected with a Quantamaster fluorometer (Photon Technology International, South Brunswick, NJ). Samples of FHS-myofibrils were excited by a 75 W xenon arc lamp through a single-grating monochromator at 470 nm. The emitted fluorescence was collected from 500 to 600 nm using a single-grating monochromator interfaced to a PMT and computer for data storage and analysis. Slit widths were 2 nm.

Solvent quenching experiments were performed at 25°C with 1–2 mg/ml FHS-myofibrils in rigor, relaxing, or activating conditions. Some experiments, as noted in the text, also included an additional 150 mM KCl. Fluorescence values were taken at the peak of the emission spectrum for each sample, usually at 525 nm. After an initial reading in the absence of quencher (F_0), quencher was added to the sample in increasing amounts and the remaining fluorescence (F) determined. Typically, 4-hydroxy-TEMPO was added in increments of 10–20 mM up to 80 mM, and potassium iodide was added in increments of 5–10 mM up to 40 mM. Ionic strength was adjusted when necessary with KCl. The final concentration of potassium iodide added was limited by its contribution to the ionic strength of our solutions. All spectra were corrected for the added volume of the quencher and inner filter effects when necessary. The relative fluorescence change F_0/F was then plotted versus the quencher concentration for each experiment to asses the accessibility of FHS at Lys-553 of myosin to the various solvent quenchers (Q). The resulting plots were fit to the Stern-Volmer relationship for dynamic solvent quenching using a linear least-squares routine in SigmaPlot (v5.0, SPSS Inc., Chicago, IL):

$$F_0/F = K_{SV}[Q] + 1 \quad (1)$$

where the slope, K_{SV}, is the Stern-Volmer constant that qualitatively describes the degree of accessibility of the fluorescent probe (FHS) to the solvent (Eftink and Ghiron, 1976). For actively contracting myofibrils, the observed Stern-Volmer constant ($K_{SV\text{-}Act}$) was assumed to be composed of two components; one arising from detached cross-bridges with a Stern-Volmer constant equal to that observed with relaxed myofibrils ($K_{SV\text{-}Rel}$), and one arising from actin-bound cross-bridges with a Stern-Volmer constant equal to that observed with rigor myofibrils ($K_{SV\text{-}Rig}$). Therefore, $K_{SV\text{-}Act}$ was assumed to be a linear combination of $K_{SV\text{-}Rig}$, weighted by the fraction of actin-bound cross-bridges (f_B), and $K_{SV\text{-}Rel}$, weighted by the fraction of detached cross-bridges ($1 - f_B$):

$$K_{SV\text{-}Act} = f_B * TK_{SV\text{-}Rig} + (1 - f_B) * K_{SV\text{-}Rel} \quad (2)$$

Thus, the fraction of actin-bound cross-bridges in actively contracting myofibrils can be determined by:

$$f_B = (K_{SV\text{-}Act} - K_{SV\text{-}Rel})/(K_{SV\text{-}Rig} - K_{SV\text{-}Rel}) \quad (3)$$

RESULTS

Labeling of myofibrils with FHS

Skeletal muscle myofibrils were selectively labeled with FHS with >75% of the total fluorescence being incorporated into the myosin heavy chain as determined by scanning densitometry (Fig. 1 *A*). Smaller amounts of fluorescence were incorporated into an unidentified large molecular weight protein, α-actinin, troponin T, tropomyosin, and light chain 1 and 2. However, as can be seen clearly in Fig. 1 *A*, actin was not labeled at all. Furthermore, limited tryptic digestion of myosin in relaxed myofibrils shows significant labeling of the 75 kD fragment of myosin subfragment 1 (S1), consistent with the primary site of modification being the 50 kD fragment containing Lys-553 (Fig. 1 *B*). The myosin rod, which is rich in lysine and included in the 150 kD tryptic fragment of myosin, was also labeled by FHS, but to a lesser extent than the 75 kD tryptic fragment of myosin containing Lys-553. Finally, rigor myofibrils were protected from trypsinolysis at the 50/20 kD junction, as evidenced by the relative lack of appearance of the 75 kD fragment (Fig. 1 *B*). Thus, myosin appears to bind normally to actin under rigor conditions in FHS-modified myofibrils.

The steady-state ATPase activities of relaxed and calcium-activated myofibrils were 0.64 ± 0.03 s^{-1} and 3.0 ± 0.4 s^{-1}, respectively (Table 1). FHS modification slightly increased the ATPase activity of relaxed myofibrils to 0.80 ± 0.04 s^{-1}, and slightly decreased the ATPase activity of calcium-activated myofibrils to 2.5 ± 0.3 s^{-1} (Table 1). Thus, FHS modification did not greatly alter the ability of myofibrils to hydrolyze ATP in a calcium-dependent manner. In fact, the effects of FHS modification on the ATPase activity of skeletal muscle myofibrils is similar to that observed for FHS-modified skeletal muscle myosin S1 (Bertrand et al., 1995; MacLean et al., 2000). EDC cross-linking increased the ATPase activity of FHS-myofibrils to 3.6 ± 0.2 s^{-1} (Table 1). This modest increase is consistent with previous studies, which have shown that only a small fraction (<25%) of the myosin heads are cross-linked to actin in this preparation (Glyn and Sleep, 1985; Duong and Reisler, 1989; Berger and Thomas, 1993). Nevertheless, this level of cross-linking was sufficient to prevent active shortening of the FHS myofibrils, as observed by phase-contrast microscopy.

Solvent quenching in rigor and relaxed FHS-myofibrils

Fluorescence quenching experiments were carried out to examine the solvent accessibility of FHS in rigor and relaxed FHS-myofibrils. These results are summarized in Table 2. The fluorescence from FHS was effectively quenched in both rigor (K_{SV} = 24.3 ± 0.3 M^{-1}) and relaxed FHS-myofibrils (K_{SV} = 27.5 ± 0.3 M^{-1}) by the neutral quencher 4-hydroxy-TEMPO at low ionic strength (Fig. 2). Potassium iodide was a less effective quencher of FHS fluorescence than 4-hydroxy-TEMPO at both low ionic strength and with the addition of high salt (150 mM KCl). At low ionic strength the binding of cross-bridges to actin under rigor conditions (K_{SV} = 2.6 ± 0.7 M^{-1}) protects a significant portion of the FHS fluorescence in myofibrils

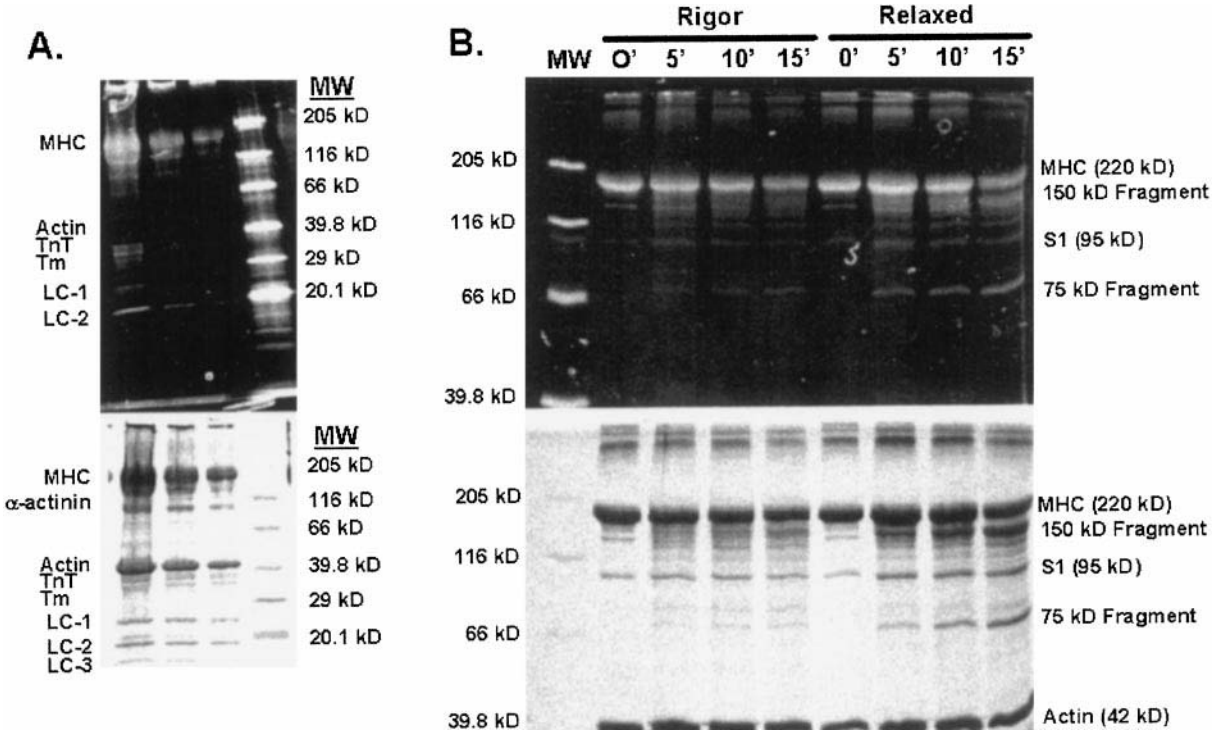

FIGURE 1 Fluorescent image (top) and Coomassie-stained image (*bottom*) of (*A*) 4–20% gradient and (*B*) 7.5% SDS-polyacrylamide gels of FHS-labeled skeletal muscle myofibrils. In (*B*) tryptic digests of rigor (*lanes 2–5*) and relaxed (*lanes 6–9*) FHS-myofibrils are shown at the right as a function of proteolysis time (see Methods). MW = molecular weight markers; MHC = myosin heavy chain; TnT = troponin T; Tm = tropomyosin; LC = myosin light chain.

from quenching by iodide relative to relaxed conditions (K_{SV} = 9.1 ± 0.5 M^{-1}) (Fig. 3). Furthermore, upon the addition of high salt (150 mM KCl), the protection offered from iodide quenching by actin binding in rigor myofibrils was virtually abolished (K_{SV} = 3.5 ± 0.6 M^{-1} in rigor FHS-myofibrils and K_{SV} = 4.2 ± 0.4 M^{-1} in relaxed FHS-myofibrils; Fig. 4). These results are quite similar to those obtained from analogous solvent quenching experiments with FHS-S1 (MacLean et al., 2000), in which Lys-553 is known to be the primary site of modification by FHS (Bertrand et al., 1995). Together, these results suggest Lys-553 of the myosin heavy chain is also the primary site of modification in FHS-myofibrils, and that this probe is not sterically protected from solvent access when myosin S1 is bound to actin in a rigor complex. It is more likely that actin is providing an electrostatic shield from the negatively charged iodide quencher (MacLean et al., 2000).

TABLE 1 Myofibrillar Mg^{2+}-ATPase activities

	Relaxed (s^{-1})	Ca^{2+}-activated (s^{-1})
Unmodified myofibrils	0.64 ± 0.03	3.0 ± 0.4
FHS-myofibrils	0.80 ± 0.04	2.5 ± 0.3
EDC-FHS-myofibrils	n.d.	3.6 ± 0.2

Myofibrillar Mg^{2+}-ATPase activities were determined as described in Methods. Skeletal muscle myofibrils were modified with FHS and/or EDC as described in Methods. All values are given as the mean ± SE (n.d. = not determined).

Solvent quenching of calcium-activated FHS-myofibrils

The accessibility of FHS to iodide quenchers in the solvent was determined at low ionic strength in calcium-activated FHS-myofibrils mildly cross-linked with EDC to prevent

TABLE 2 Solvent quenching of FHS-myofibrils

	K_{SV} (M^{-1})	f_B
4-hydroxy-TEMPO (0 mM KCl)		
Relaxed	27.5 ± 0.3	—
Rigor	24.3 ± 0.3	—
Potassium iodide (150 mM KCl)		
Relaxed	4.2 ± 0.4	—
Rigor	3.5 ± 0.6	—
Potassium iodide (0 mM KCl)		
Relaxed	9.1 ± 0.5	—
Rigor	2.6 ± 0.7	—
Ca^{2+}-activated*	7.2 ± 0.6	0.29 ± 0.12

K_{SV}, the Stren-Volmer quenching constant, was determined using Eq. 1. f_B, the fraction of actin-bound cross-bridges, was determined using Eq. 3. *Ca^{2+}-activated myofibrils were mildly cross-linked to prevent shortening. Values are given as the mean ± SE.

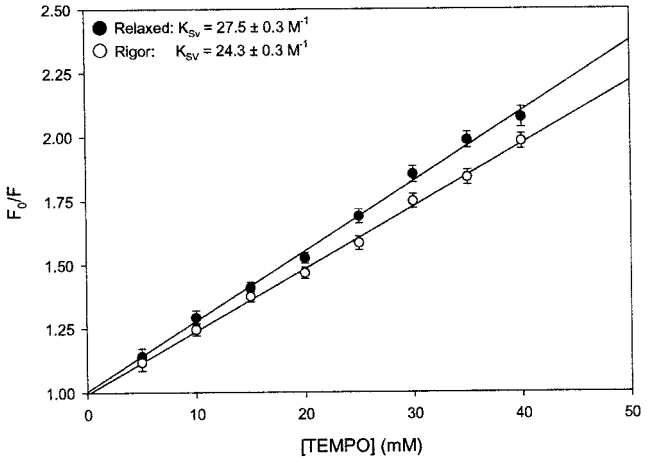

FIGURE 2 Stern-Volmer plots of relaxed (●) and rigor (○) FHS-myofibrils quenched by 4-hydroxy-TEMPO at low ionic strength (0 mM KCl). K_{SV}, the Stern-Volmer quenching constant, was determined using Eq. 1. Values are given as the mean ± SE.

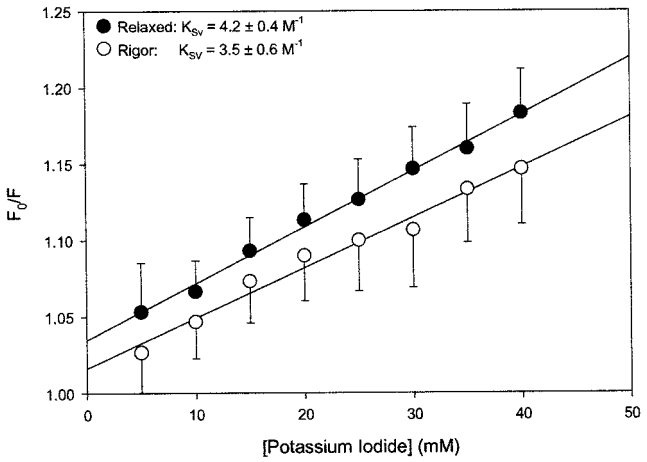

FIGURE 4 Stern-Volmer plots of relaxed (●) and rigor (○) FHS-myofibrils quenched by potassium iodide at high ionic strength (150 mM KCl). K_{SV}, the Stern-Volmer quenching constant, was determined using Eq. 1. Values are given as the mean ± SE.

shortening (summarized in Table 2). There was no significant difference in the accessibility of FHS probes in cross-linked versus uncross-linked myofibrils under rigor and relaxed conditions (data not shown). FHS probes in calcium-activated myofibrils were quenched to an intermediate degree (K_{SV} = 7.2 ± 0.6 M^{-1}) between rigor and relaxed conditions by potassium iodide at low ionic strength (Fig. 5), indicating that a fraction of the probes are protected from iodide quenching by actin during active cycling of the cross-bridges. Analysis of these data (Eq. 3) suggests that only 29 ± 12% of the cross-bridges are bound to actin in isometrically contracting FHS-myofibrils.

DISCUSSION

Summary of results

Skeletal muscle myofibrils were labeled predominantly on the myosin heavy chain with the fluorescent probe FHS. The neutral solvent quencher 4-hydroxy-TEMPO effectively quenched the FHS fluorescence in both rigor and relaxed myofibrils at low ionic strength, while the negatively charged solvent quencher iodide effectively quenched the FHS fluorescence in relaxed myofibrils but not rigor myofibrils at low ionic strength. However, the protection from iodide quenching in rigor myofibrils was abolished at higher salt concentrations (150 mM KCl). Together, these

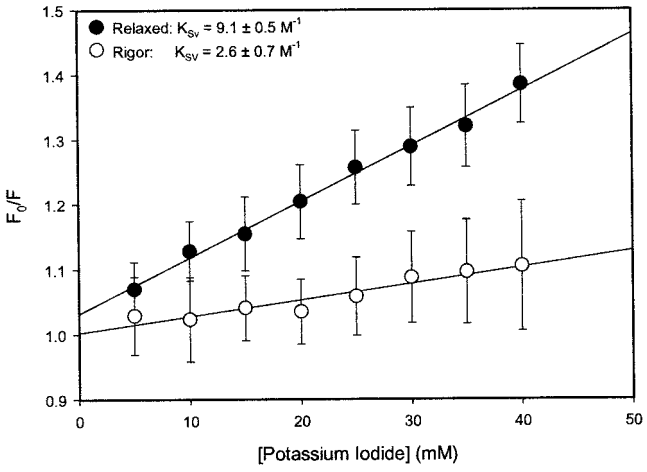

FIGURE 3 Stern-Volmer plots of relaxed (●) and rigor (○) FHS-myofibrils quenched by potassium iodide at low ionic strength (0 mM KCl). K_{SV}, the Stern-Volmer quenching constant, was determined using Eq. 1. Values are given as the mean ± SE.

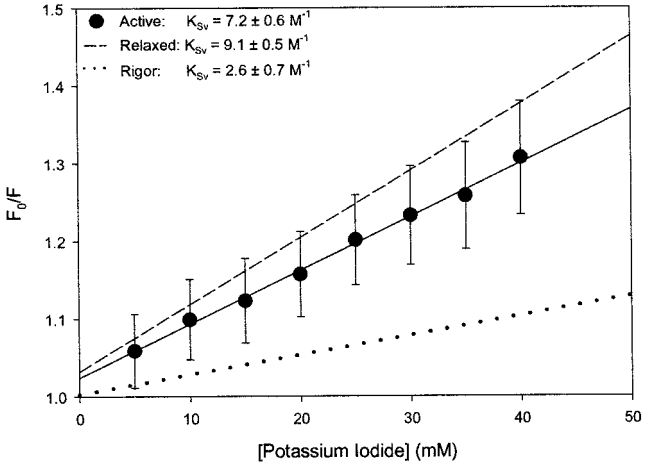

FIGURE 5 Stern-Volmer plots of active (●) FHS-myofibrils quenched by potassium iodide at low ionic strength (0 mM KCl). Shown for comparison are the fits to the relaxed (- - -) and rigor (· · ·) data at low ionic strength (0 mM KCl) from Fig. 3. K_{SV}, the Stern-Volmer quenching constant, was determined using Eq. 1. Values are given as the mean ± SE.

results suggest that the fluorescence behavior of FHS-myofibrils is similar to that of FHS-S1 (MacLean et al., 2000), and that as with FHS-S1, Lys-553 is the predominant residue labeled on the myosin heavy chain (Bertrand et al., 1995). Finally, iodide quenching of calcium- activated FHS-myofibrils, mildly cross-linked with EDC to prevent shortening, indicates that 29 ± 12% of the myosin heads are strongly bound to actin during isometric contractions at low ionic strength.

Fluorescent labeling of FHS-myofibrils

Myofibrils labeled with FHS were predominantly modified on the myosin heavy chain, with virtually no fluorescence incorporated into actin. An unidentified large molecular weight protein, α-actinin, troponin T, tropomyosin, and light chain 1 and 2 were all also labeled by FHS, but to a much lesser extent than the myosin heavy chain. Both the S1 and rod portions of myosin were labeled by FHS. Given that Lys-553 is the primary site of FHS incorporation in purified skeletal muscle myosin S1 (Bertrand et al., 1995), it is likely to be the primary site of modification within the S1 portion of myosin in myofibrils as well. Consistent with this interpretation is the solvent quenching results from the FHS-modified myofibrils. The fluorescence from both relaxed and rigor FHS-myofibrils is effectively quenched by 4-hydroxy-TEMPO at low ionic strength and iodide at physiological ionic strength (150 mM KCl). However, at low ionic strength the fluorescence from relaxed FHS-myofibrils is effectively quenched by iodide, while the fluorescence from rigor FHS-myofibrils is not. The observed patterns of solvent quenching in FHS-myofibrils are similar to those observed in FHS-labeled myosin S1 in solution, in which FHS-S1 alone is analogous to the relaxed state in FHS-myofibrils and FHS-S1 bound to actin in the absence of nucleotide is analogous to the rigor state in FHS-myofibrils (MacLean et al., 2000). The other major site of modification by FHS in skeletal muscle myofibrils appears the to be the myosin rod. It is not known which residue(s) are labeled by FHS in the myosin rod, but given that this portion of myosin is relatively rich in lysines, this is not an unexpected result. The presence of FHS probes outside of the myosin head does not greatly alter the interpretation of experiments from calcium-activated FHS-myofibrils, and most likely is responsible for the small amount of background fluorescence that is insensitive to cross-bridge binding to actin in the rigor experiments.

Effects of FHS modification and EDC cross-linking on myofibrillar function

Modification of skeletal muscle myofibrils by FHS does slightly alter the enzymatic properties of myosin. The steady-state ATPase activity of relaxed myofibrils was slightly increased by FHS labeling (0.64 ± 0.03 s^{-1} to 0.80 ± 0.04 s^{-1}), while the steady-state ATPase activity of calcium-activated myofibrils was slightly decreased by FHS modification (3.0 ± 0.4 s^{-1} to 2.5 ± 0.3 s^{-1}). These results are consistent with effects of FHS modification on purified skeletal muscle myosin S1 in solution (Bertrand et al., 1995; MacLean et al., 2000), in which the Mg^{2+}-ATPase activity is slightly elevated and the actin-activated Mg^{2+}-ATPase activity is slightly depressed. The magnitude of the effects on ATPase activity in the FHS-myofibrils is reduced compared to that in FHS-S1, but this is probably due to a lower level of FHS modification at Lys-553 in the myofibril preparation. While it is not possible to accurately determine the level of FHS incorporation at Lys-553 in the myofibril preparation, it is unlikely that it reaches the level of >80% found in FHS-S1 (Bertrand et al., 1995; MacLean et al., 2000). Finally, FHS modification did not appear to alter myosin ability to bind strongly to actin in the rigor state, as evidenced by the lack of proteolytic susceptibility of the 50/20 kD junction in rigor FHS-myofibrils relative to the relaxed state.

EDC-cross-linked myofibrils were originally introduced as a solution model for isometrically contracting muscle fibers by Glyn and Sleep in 1985. Since then, this preparation has proven to be an excellent system for examining cross-bridge binding (Duong and Reisler, 1989), rotational dynamics (Berger and Thomas, 1993), and ATPase kinetics (Herrmann et al., 1993) in the myofilament lattice under isometric conditions. The mild cross-linking conditions in this preparation results in only a small fraction of the myosin heads being cross-linked to actin (<20%), and the sarcomeres are prevented from shortening in the presence of millimolar calcium and MgATP as determined by phase contrast microscopy (Glyn and Sleep, 1985; Duong and Reisler, 1989; Berger and Thomas, 1993). Consistent with previous results using non-fluorescent, EDC-cross-linked myofibrils (Glyn and Sleep, 1985; Duong and Reisler, 1989; Berger and Thomas, 1993), the calcium-activated Mg^{2+}-ATPase activity of EDC-cross-linked FHS-myofibrils was almost 50% greater than that of uncross-linked FHS-myofibrils (3.6 ± 0.2 s^{-1} to 2.5 ± 0.3 s^{-1}). Thus, EDC-cross-linked FHS-modified skeletal muscle myofibrils appear to be a valid model system for examining cross-bridge binding within the myofilament lattice during calcium-activated, isometric contractions.

Fraction of actin-bound cross-bridges in isometrically contracting myofibrils

The fraction of myosin heads strongly bound to actin in calcium-activated, EDC-cross-linked, FHS-myofibrils was determined to be 29 ± 12% at low ionic strength by comparison to the degree of iodide quenching in relaxed and rigor FHS-myofibrils using Eq. 3. This calculation is valid assuming 1) iodide quenching of FHS in relaxed myofibrils

is indicative of detached cross-bridges, 2) iodide quenching of FHS in rigor myofibrils is indicative of strongly bound cross-bridges, 3) strongly bound cross-bridges in calcium-activated myofibrils behave similarly as strongly bound cross-bridges in rigor myofibrils with respect to iodide quenching of FHS, and 4) the fluorescence from FHS probes in the presence of solvent quenchers in calcium-activated myofibrils is a linear combination of the fluorescence arising from the detached and actin-bound cross-bridges. The last assumption is certainly valid, because each population of FHS probes, those on actin-bound or detached myosin heads, interacts with the iodide ions in the solvent independently. Inasmuch as solvent quenching affects only those probes in the excited state, which has a nanosecond lifetime, only those probes on myosin heads that are bound to actin are likely to be protected from iodide. This, of course, also assumes that we are primarily examining dynamic, and not static, quenching events. However, the Stern-Volmer plots are relatively linear and do not suggest the presence of a significant static quenching component.

The assumptions that the FHS fluorescence of relaxed myofibrils is indicative of detached cross-bridges, and that the FHS fluorescence of rigor myofibrils is indicative of strongly bound cross-bridges, are probably valid given the similarity between the solvent quenching behavior of these preparations with analogous experiments using purified actin and FHS-S1 (MacLean et al., 2000). The similarity of these results also suggests that we are primarily observing fluorescence from FHS probes at Lys-553 of the myosin heavy chain. The assumption that FHS probes on actin-attached myosin heads in actively contracting myofibrils behave similarly to those in actin-bound cross-bridges in rigor myofibrils is the most speculative of our assumptions. Therefore, the possibility exists that we are under- or over-estimating the fraction of myosin heads strongly bound to actin in calcium-activated myofibrils, if the Stern-Volmer quenching constant for actively bound cross-bridges is actually higher or lower than that of rigor bound cross-bridges. However, it is likely that myosin must adopt a strongly bound conformation similar to that observed in rigor in order to generate and maintain force during an isometric contraction. Furthermore, intrinsic fluorescence from Trp-546, located on the same α-helix as Lys-553 of the myosin heavy chain, indicates that this region interacts directly with actin in the strongly bound state and forms a stable complex in both the ADP-rigor and rigor states (Yengo et al., 1998; 1999). Thus, the value of 29% for the fraction of strongly bound myosin heads in the calcium-activated FHS-myofibrils by iodide quenching is probably a reasonable estimate.

Other studies have attempted to determine the fraction of actin-bound cross-bridges (f_B) in actively contracting, isometric muscle fibers and myofibrils in the past. Mechanical stiffness measurements have suggested that as much as 80% of the cross-bridges may be bound to actin in a strongly bound state during isometric contractions (Goldman and Simmons, 1977). However, this is likely to be an overestimate given that high-resolution x-ray diffraction (Huxley et al., 1994; Wakabayashi et al., 1994) and mechanical (Higuchi et al., 1995) studies indicate that more than half of the sarcomere's compliance may lie outside of the cross-bridge in the thick and thin filaments. More recent estimates from mechanical stiffness measurements place the value for the fraction of actin-bound cross-bridges in isometric muscle fibers at 43% (Linari et al., 1998). However, 43% is still significantly higher than estimates of f_B made from EPR spectroscopy and proteolytic digestion experiments, which are in the range of 20–25%. Cooke et al. (1982) observed that 20% of the myosin heads in a spin-labeled, isometrically contracting muscle fiber have a similar orientation to cross-bridges in a rigor muscle fiber, while the other 80% are identical to the detached cross-bridges in relaxed muscle fibers. There may, in fact, be fewer strongly bound cross-bridges in the spin-labeled muscle fibers due to alterations in the kinetics of the ATPase cycle in SH-1 modified myosins (Ostap et al., 1993). However, Duong and Reisler (1989) showed that 25% of the myosin heads in calcium-activated EDC-cross-linked myofibrils, which are not modified at SH-1, are protected from tryptic digestion at the 50/20 kD junction in the myosin heavy chain. Berger and Thomas (1993) confirmed the result of Duong and Reisler (1989) and showed that this value increases to 37% at low ionic strength, presumably due to additional contributions from weakly bound cross-bridges.

There is a possible resolution to the discrepancy between the more recent stiffness measurements (f_B = 43%) and the EPR and proteolytic digestion studies (f_B = 20–25%). Tryptic susceptibility of the 50/20 kD junction and spectroscopic probes are sensitive to the interaction of individual myosin heads with actin, while stiffness measurements are only sensitive to the interaction of the entire cross-bridge (i.e., double-headed myosin molecules) with actin. Thus, if the compliance within the cross-bridge lies outside of the myosin head (for example in the S2 region), and each cross-bridge binds to actin with one of its two heads, then 40% of the cross-bridges could be bound to actin with only 20% of the total number of myosin heads. However, if any of the cross-bridge compliance resides within the S1 portion of the myosin molecule itself, then the stiffness results are still in contrast to the proteolytic digestion and spectroscopic data.

Our current results (f_B = 29%) are in between the estimates from mechanical stiffness measurements (f_B = 43%) and those from other techniques (f_B = 20–25%). Although the error in our estimation is relatively high (±12%), it is possible that the estimates from different techniques reflect real differences in the fraction of actin-bound myosin heads in skeletal muscle myofibrils. One possible explanation may be the fact that we, by necessity for the iodide quenching effects, carried our experiments out at low ionic strength, while most of the previous studies were performed at more

physiological ionic strengths (150–200 mM). Berger and Thomas (1993) found a higher fraction of actin-bound cross-bridges in isometrically contracting EDC-cross-linked myofibrils at low ionic strength (f_B = 37%) than at physiological ionic strength (f_B = 24%). This difference could be due to the presence of more weakly and/or strongly bound cross-bridges at low ionic strength than at physiological ionic strength. Protection from proteolytic digestion of the 50/20 kD junction in myosin by actin can be sensitive to weakly bound as well as strongly bound cross-bridges. However, FHS-S1 is not protected by actin from iodide quenching in the weakly bound states (MacLean et al., 2000), and this is therefore likely to be true in FHS-myofibrils as well. Therefore, the estimate of 29% obtained in this work probably represents myosin heads bound to actin in a strongly bound complex during isometric contractions within the myofilament lattice at low ionic strength, and this value would likely decrease to 20–25% with increasing ionic strength. The 9% discrepancy between the value of f_B measured in this work (29%) and that measured previously by Berger and Thomas (1993) under similar conditions (37%) is likely due to the sensitivity of proteolytic digestion experiments to the presence of weakly bound cross-bridges. Clearly, however, a real-time assay for the detection of actin-bound myosin heads in myofilament lattice of muscle fibers and myofibrils is advantageous to indirect mechanical measurements that cannot differentiate between singly bound myosin heads and doubly bound myosin cross-bridges.

Future directions

Conventional in vitro motility assays have been used to estimate the duty cycle (i.e., the fraction of the ATPase cycle in which myosin interacts strongly with actin) during unloaded filament sliding. Several groups (Uyeda et al., 1990; Harris and Warshaw, 1993) have reported low duty cycle estimates (<5%), consistent with a tight chemomechanical coupling model in which the hydrolysis of one molecule of ATP by myosin results in a single productive interaction with actin. Measurements of unitary displacements and forces from single myosin heads using optical laser traps (Finer et. al, 1994; Molloy et al., 1995; Guilford et al., 1997) have also been consistent with tight coupling between the mechanical and chemical ATPase cycles of myosin. Conversely, evidence for a loosely coupled chemomechanical cycle, in which the hydrolysis of one molecule of ATP by myosin results in multiple productive interactions with actin, has been obtained from mechanical in vitro studies, both in solution and in single muscle fibers. Estimates of the distance a single cross-bridge can interact with the thin filament per ATP hydrolyzed during isotonic shortening has been measured in single muscle fibers and found to be greater than the size of the myosin head or actin monomer (Higuchi and Goldman, 1991; 1995). The interaction distance measured in these experiments, however, is directly proportional to cross-bridge attachment, which was determined by traditional stiffness measurements. Therefore, if the stiffness measurements do indeed over-estimate the fraction of actin-bound cross-bridges in actively contracting muscle fibers due to myofilament compliance, then the estimates of interaction distance will be over-estimated by the same amount. In solution, force fluctuations of a few myosin molecules interacting with a single actin filament have been shown to be high under large (near isometric) loads, suggesting a low duty cycle, but small under unloaded conditions, suggesting a high duty cycle (Ishijima et al., 1991). This is consistent with the high estimates of duty cycle (80%) during filament sliding in the in vitro motility assay by Harada et al. (1990). Yanagida's laboratory has also demonstrated that a single myosin head can make repeated powerstrokes per ATP molecule hydrolyzed (Kitamura et al., 1999) and that the hydrolysis of ATP and the myosin powerstroke can be temporally uncoupled from each other (Ishijima et al., 1998). These results are in direct contrast with conventional models of muscle contraction in which the biochemical and mechanical contractile cycles are tightly coupled.

The technique developed in this work and the accompanying paper (MacLean et al., 2000) may be useful for addressing the current controversies involving the chemomechanical coupling ratio during unloaded myofilament sliding. Because iodide quenching of FHS-S1 and FHS-myofibrils at low ionic strength is sensitive to strong binding interactions between actin and myosin, it should be possible to directly examine the duty cycle in both isotonically shortening muscle fibers labeled with FHS, and during unloaded actin filament sliding in conventional in vitro motility assays using FHS-labeled whole myosin. Solvent quenching is an excited state reaction and occurs on the nanosecond time scale, and thus such time-resolved experiments should be achievable. Experiments could be done at an intermediate iodide concentration in which the fluorescence from actin-bound and detached myosin heads is significantly different. In single muscle fibers this signal could easily be calibrated as function of isometric force, varying either the thick and thin filament overlap (i.e., sarcomere length) or the concentration of activating calcium. The fact that myosin appears to be predominantly labeled at Lys-553 in skeletal muscle myofibrils suggests that this technique can be extended to purified myosin for the in vitro motility assays as well as to single, skinned muscle fibers. We have already demonstrated that the duty cycle for isometrically contracting skeletal muscle myofibrils can be accurately determined (f_B = 29%). The challenge remains to make similar estimates for conditions other than isometric in order to answer critical questions remaining about energy transduction during muscle contraction.

The authors thank Andrew Wellman for excellent technical help with this project, as well as the University of Vermont Muscle Club for many stimulating discussions.

This work was supported by National Institutes of Health Grant AR44219 and a grant from the American Heart Association (to C.L.B.).

REFERENCES

Berger, C. L., J. S. Craik, D. R. Trentham, J. E. T. Corrie, and Y. E. Goldman. 1996. Fluorescence polarization of skeletal muscle fibers labeled with rhodamine isomers of the myosin heavy chain. *Biophys. J.* 71:3330–3343.

Berger, C. L., and D. D. Thomas. 1993. Rotational dynamics of actin-bound myosin heads in active myofibrils. *Biochemistry.* 32:3812–3821.

Bertrand, R., J. Derancourt, and R. Kassab. 1995. Production and properties of skeletal myosin subfragment 1 selectively labeled with fluorescein at Lysine-553 proximal to strong actin-binding site. *Biochemistry.* 34:9500–9507.

Cooke, R., M. S. Crowder, and D. D. Thomas. 1982. Orientation of spin labels attached to cross-bridges in contracting muscle fibers. *Nature.* 300:776–778.

Duong, A. M., and E. Reisler. 1989. Binding of myosin to actin in myofibrils during ATP hydrolysis. *Biochemistry.* 28:1307–1313.

Eftink, M. R., and C. A. Ghiron. 1976. Exposure of tryptophanyl residues in proteins. Quantitative determination by fluorescence quenching studies. *Biochemistry.* 15:672–680.

Finer, J. T., R. M. Simmons, and J. A. Spudich. 1994. Single myosin molecule mechanics: piconewton forces and nanometre steps. *Nature.* 368:113–119.

Glyn, H., and J. Sleep. 1985. Dependence of adenosine triphosphatase activity of rabbit psoas muscle fibres and myofibrils on substrate concentration. *J. Physiol.* 365:259–276.

Goldman, Y. E., and R. M. Simmons. 1977. Active and rigor muscle stiffness. *J. Physiol.* 269:55P–57P.

Greene, L. E., and E. Eisenberg. 1980. The binding of heavy meromyosin to F-actin. *J. Biol. Chem.* 255:549–554.

Guilford, W. H., D. E. Dupuis, G. Kennedy, J. Wu, J. B. Patlak, and D. M. Warshaw. 1997. Smooth muscle and skeletal muscle myosins produce similar unitary forces and displacements in the laser trap. *Biophys. J.* 72:1006–1021.

Harada, Y., K. Sakurada, T. Aoki, D. D. Thomas, and T. Yanagida. 1990. Mechanochemical coupling in actomyosin energy transduction studied by in vitro movement assay. *J. Mol. Biol.* 216:49–68.

Harris, D. E., and D. M. Warshaw. 1993. Smooth and skeletal muscle myosin both exhibit low duty cycles at zero load in vitro. *J. Biol. Chem.* 268:14764–14768.

Herrmann, C., J. Sleep, P. Chaussepied, F. Travers, and T. Barman. 1993. A structural and kinetic study on myofibrils prevented from shortening by chemical cross-linking. *Biochemistry* 32:7255–7263.

Higuchi, H., and Y. E. Goldman. 1991. Sliding distance between actin and myosin filaments per ATP molecule hydrolysed in skinned muscle fibres. *Nature.* 352:352–354.

Higuchi, H., and Y. E. Goldman. 1995. Sliding distance per ATP molecule hydrolyzed by myosin heads during isotonic shortening of skinned muscle fibers. *Biophys. J.* 69:1491–1507.

Higuchi, H., T. Yanagida, and Y. E. Goldman. 1995. Compliance of thin filaments in skinned fibers of rabbit skeletal muscle. *Biophys. J.* 69: 1000–1010.

Huxley, H. E., A. Stewart, H. Sosa, and T. Irving. 1994. X-ray diffraction measurements of the extensibility of actin and myosin filaments in contracting muscle. *Biophys. J.* 67:2411–2421.

Ishijima, A., T. Doi, K. Sakurada., and T. Yanagida. 1991. Sub-piconewton force fluctuations of actomyosin in vitro. *Nature.* 352:301–306.

Ishijima, A., H. Kojima, T. Funatsu,, M. Tokunaga, H. Higuchi, H. Tanaka, and T. Yanagida. 1998. Simultaneous observation of individual ATPase and mechanical events by a single myosin molecule during interaction with actin. *Cell.* 92:161–171.

Kitamura, K., M. Tokunaga, A. H. Iwane, and T. Yanagida. 1999. A single myosin head moves along an actin filament with regular steps of 5.3 nanometres. *Nature.* 397:129–134.

Knight, P. J., and J. A. Trinick. 1982. Preparation of myofibrils. *Methods Enzymol.* 85(B):9–12.

Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature.* 227:680–685.

Lanzetta, P. A., L. J. Alvarez, P. S. Reinach, and O. A. Candia. 1979. An improved assay for nanomole amounts of inorganic phosphate. *Anal. Biochem.* 100:95–97.

Linari, M., I. Dobbie, M. Reconditi, N. Koubassova, M. Irving, G. Piazzesi, and V. Lombardi. 1998. The stiffness of skeletal muscle in isometric contraction and rigor: the fraction of myosin heads bound to actin. *Biophys. J.* 74:2459–2473.

Ludescher, R. D., and D. D. Thomas. 1988. Microsecond rotational dynamics of phosphorescently-labeled muscle cross-bridges. *Biochemistry.* 27:3343–3351.

MacLean, J. J., L. R. Chrin, and C. L. Berger. 2000. Dynamics at Lys-553 of the acto-myosin interface in the weakly and strongly bound states. *Biophys. J.* 78:1441–1448.

Mendelson, R., and E. P. Morris. 1997. The structure of the acto-myosin subfragment 1 complex: Results of searches using data from electron microscopy and x-ray crystallography. *Proc. Natl. Acad. Sci. USA.* 94:8533–8538.

Milligan, R. A. 1996. Protein-protein interactions in the rigor actomyosin complex. *Proc. Natl. Acad. Sci. USA.* 93:21–26.

Molloy, J. E., J. E. Burns, J. Kendrick-Jones, R. T. Tregear, and D. C. White. 1995. Movement and force produced by a single myosin head. *Nature.* 378:209–212.

Ostap, E. M., H. D. White, and D. D. Thomas. 1993. Transient detection of spin-labeled myosin subfragment-1 conformational states during ATP hydrolysis. *Biochemistry.* 32:6712–6720.

Rayment, I., H. M. Holden, M. Whittaker, C. B. Yohn, M. Lorenz, K. C. Holmes, and R. A. Milligan. 1993. Structure of the actin-myosin complex and its implications for muscle contraction. *Science.* 261:58–65.

Stein, L. A., R. P. Schwarz, Jr., P. B. Chock, and E. Eisenberg. 1979. Mechanism of actomyosin adenosine triphosphatase. Evidence that adenosine 5′-triphosphate hydrolysis cam occur without dissociation of the actomyosin complex. *Biochemistry.* 18:3895–3909.

Uyeda, T. Q., S. J. Kron, and J. A. Spudich. 1990. Myosin step size. Estimation from slow sliding movement of actin over low densities of heavy meromyosin. *J. Mol. Biol.* 214:699–710.

Wakabayashi, K., Y. Sugimoto, H. Tanaka, Y. Ueno, Y. Takezawa, and Y. Amemiya. 1994. X-ray diffraction evidence for the extensibility of actin and myosin filaments during muscle contraction. *Biophys. J.* 67: 2422–2435.

Yengo, C. M., L. Chrin, A. S. Rovner, and C. L. Berger. 2000. Intrinsic tryptophan fluorescence identifies specific conformational changes at the acto-myosin interface upon actin binding and ADP release. *Biochemistry*. 38:14515–14523.

Yengo, C. M., P. M. Fagnant, L. Chrin, A. S. Rovner, and C. L. Berger. 1998. Smooth muscle myosin mutants containing a single tryptophan reveal molecular interactions at the actin-binding interface. *Proc. Natl. Acad. Sci. USA*. 95:12944–12949.