Dynamics at Lys-553 of the Acto-Myosin Interface in the Weakly and Strongly Bound States

Jeffrey J. MacLean, 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 Lys-553 of skeletal muscle myosin subfragment 1 (S1) was specifically labeled with the fluorescent probe FHS (6-[fluorescein-5(and 6)-carboxamido]hexanoic acid succinimidyl ester) and fluorescence quenching experiments were carried out to determine the accessibility of this probe at Lys-553 in both the strongly and weakly actin-bound states of the MgATPase cycle. Solvent quenchers of varying charge [nitromethane, (2,2,6,6-tetramethyl-1-piperinyloxy) (TEMPO), iodide (I^-), and thallium (Tl^+)] were used to assess both the steric and electrostatic accessibilities of the FHS probe at Lys-553. In the strongly bound rigor (nucleotide-free) and MgADP states, actin offered no protection from solvent quenching of FHS by nitromethane, TEMPO, or thallium, but did decrease the Stern-Volmer constant by almost a factor of two when iodide was used as the quencher. The protection from iodide quenching was almost fully reversed with the addition of 150 mM KCl, suggesting this effect is ionic in nature rather than steric. Conversely, actin offered no protection from iodide quenching at low ionic strength during steady-state ATP hydrolysis, even with a significant fraction of the myosin heads bound to actin. Thus, the lower 50 kD subdomain of myosin containing Lys-553 appears to interact differently with actin in the weakly and strongly bound states.

INTRODUCTION

At the molecular level muscle contraction is driven by the cyclic interaction of two filamentous proteins, myosin and actin. The globular portion of myosin (subfragment 1 or S1) is an enzyme that utilizes the hydrolysis of ATP to provide both the chemical energy needed for muscle contraction and a means of mediating the affinity between actin and myosin during the contractile cycle. In the absence of ATP myosin binds tightly to actin in a rigor complex, but rapidly dissociates from the thin filament upon binding ATP. After the hydrolysis of ATP to form ADP and inorganic phosphate (P_i), myosin can rebind to actin in a rapid-equilibrium, weakly bound state. Release of P_i is thought to result in a large conformational change in myosin in which the molecule increases its affinity for actin by several orders of magnitude and goes through a "powerstroke" to generate force and/or motion within the muscle fiber. Thus the resulting myosin · ADP state, like the rigor state, remains tightly bound to actin until the next ATPase cycle is initiated by the binding of a new molecule of ATP.

The atomic structures of myosin S1 (Rayment et al., 1993a; Dominguez et al., 1998; Houdusse et al., 1999) and the actin monomer (Kabsch et al., 1990) have provided valuable insights as to how the hydrolysis of ATP and subsequent product release may give rise to the conformational changes in myosin required to bind tightly to actin and generate force and/or myofilament sliding. Specifically, conformational changes at the active site are thought to be propagated both back to the long α-helical neck of the myosin molecule, which acts as a mechanical lever arm during the powerstroke, and to the front of the molecule containing the actin-binding interface. The putative actin-binding interface is split by a large cleft, the opening and closing of which may be responsible for mediating the affinity by actin and myosin (Rayment et al., 1993b). Attempts have been made to further define the actin-binding interface of myosin by using three-dimensional reconstructions of cryoelectron micrographs of myosin subfragment 1 (S1) bound to F-actin to "dock" the atomic structures of S1 and actin together in a rigor complex (Rayment et al., 1993b; Milligan, 1996; Mendelson and Morris, 1997). The resulting models postulate that myosin binds to actin in a sequential, multi-step process, in which myosin first binds to actin in a weakly bound, rapid equilibrium state, followed by the formation of a strongly bound, stereospecific complex. The weakly bound state is believed to involve a primarily ionic interaction between the positively charged, flexible surface loop that links the 50 kD and 20 kD tryptic fragments of the myosin heavy chain and the negatively charged N-terminus of actin. The strongly bound state is thought to involve at least two major structural subdomains within myosin, a helix-loop-helix motif located in the lower half of the 50 kD tryptic subdomain of the myosin heavy chain, and a surface loop that extends out from the upper half of the 50 kD tryptic subdomain of the myosin heavy chain. The lower 50 kD subdomain of myosin is thought to bind to actin first and involve both ionic and stereospecific hydrophobic components, followed by stereospecific components from the upper 50 kD subdomain. However, most of the specific interactions between actin and myosin proposed in this model are speculative, and require experimen-

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.

0006-3495/00/03/1441/08 $2.00

tal verification or refutation for the molecular details of the acto-myosin interface to be completely understood.

Of the three putative actin-binding subdomains within myosin, the flexible loop at the 50/20 kD junction is the best studied and characterized. There is extensive literature demonstrating that this site is protected from proteolytic cleavage by actin (Mornet et al., 1979), and that alterations in this loop have an impact upon the actin-activated ATPase and actin-binding activities of myosin (Uyeda et al., 1994; Rovner et al., 1995). The roles of the upper and lower 50 kD subdomains of myosin S1 within the actin-binding interface are less clear, with virtually no experimental evidence indicating the upper 50 kD subdomain is involved with actin binding at all. Indirect evidence that the lower 50 kD subdomain of myosin forms an important part of the acto-myosin interface has been obtained by Onishi et al. (1995), who demonstrated that mutating two hydrophobic amino acids in this region to more polar residues (W546S and F547H) results in greatly depressed actin-activated ATPase activities. Furthermore, Bertrand et al. (1995) have shown that Lys-553 of myosin S1 could be specifically labeled with the fluorescent probe FHS (6-[fluorescein-5(and 6)-carboxamidohexanoic acid succinimidyl ester) and that this reaction could be blocked when myosin was bound to actin in a rigor complex.

To more directly examine the role of the helix-loop-helix motif in the lower 50 kD subdomain of skeletal muscle myosin S1 in forming the tightly bound complex with actin, we have labeled Lys-553 with FHS as described by Bertrand et al. (1995). Lys-553 is in the middle of the second of the two helices (residues Asp-547 to His-558) in this putative interface region of myosin and has been postulated to bind directly to actin (Rayment et al., 1993b; Milligan, 1996; Mendelson and Morris, 1997). In the current study we have used solvent quenchers that vary in charge (nitromethane, TEMPO, thallium chloride, and potassium iodide) to directly probe the accessibility and electrostatic environment around Lys-553 of myosin when bound to actin in both the strongly (i.e., rigor (nucleotide-free) and in the presence of MgADP) and weakly (i.e., during steady-state ATP hydrolysis) bound states of the MgATPase cycle.

METHODS

Chemicals and solutions

FHS was purchased from Molecular Probes (Eugene, OR). TEMPO (2,2,6,6-tetramethyl-1-piperinyloxy), nitromethane, potassium iodide, and thallium acetate were all purchased from Aldrich (Milwaukee, WI). ATP, ADP, and all other reagents (at least analytical grade and of the highest purity possible) were purchased from Sigma (St. Louis, MO).

Proteins

Papain myosin subfragment 1 (S1) was prepared from rabbit skeletal muscle as described previously (Margossian and Lowey, 1982) and labeled at Lys-553 with FHS by the method of Bertrand et al. (1995). Concentrations of unmodified S1 were determined spectrophotometrically at 280 nm using an extinction coefficient of 0.75 $(mg/ml)^{-1}\ cm^{-1}$. Concentrations of FHS-modified S1 were determined in an identical manner after correcting the absorbance at 280 nm for contribution of the dye ($\epsilon = 2.04 \times 10^4\ M^{-1}\ cm^{-1}$). FHS concentrations were determined spectrophotometrically at 495 nm using an extinction coefficient of $68 \times 10^4\ M^{-1}\ cm^{-1}$. Rabbit skeletal F-actin was prepared by the method of Pardee and Spudich (1982), and concentrations of G-actin were determined spectrophotometrically at 290 nm using an extinction coefficient of 0.63 $(mg/ml)^{-1}\ cm^{-1}$. Protein purity was assessed using SDS-polyacrylamide gel electrophoresis by the method of Laemmli (1970). Limited tryptic digestion (trypsin/S1 ratio of 1:100 wt/wt for 10 min at 25°C) was carried out on samples of FHS-S1 to ensure that the 50 kD subdomain containing Lys-553 was selectively labeled. Tryptic digests were subsequently analyzed by SDS-polyacrylamide gel electrophoresis and visualized for fluorescence on a UV light box.

Actin-activated ATPase and actin cosedimentation assays

Actin-activated ATPase assays were performed at 25°C in 10 mM MOPS, 5 mM MgATP, 3 mM $MgCl_2$, 25 mM EGTA, pH 7.1; 0.05–0.10 mg/ml of S1 or FHS-S1 was assayed with 0–80 μM F-actin, and the rate of phosphate production measured colorimetrically by the method of Lanzetta et al. (1979). Rates of ATP hydrolysis were plotted versus actin concentration and fit to Michaelis-Menten kinetics using a nonlinear least-squares routine in SigmaPlot (v5.0, SPSS Inc., Chicago, IL).

Actin cosedimentation assays were performed by incubating 1–2 μM S1 or FHS-S1 with varying concentrations of actin under rigor conditions (10 mM MOPS, 8 mM $MgCl_2$, 25 mM EGTA, pH 7.1 at 25°C) for 30 min. Some experiments, as noted in the text, also included 2 mM MgADP or 20 mM MgATP. F-actin concentrations varied from 5 to 10 μM for the rigor and MgADP experiments to 10–80 μM for the MgATP experiments. Samples were then centrifuged for 30 min at 95,000 rpm in a Beckman TL-100 tabletop ultracentrifuge using a TLA 120.2 rotor. The resulting supernatant was removed, and the pellet was washed with 10 mM MOPS, pH 7.1 and resuspended in SDS-PAGE sample buffer in a volume equal to that of the supernatant. In addition to samples of the supernatant and pellet, equal amounts of the sample taken before centrifugation were subjected to SDS-PAGE. Assay results were assessed by visual inspection of Coomassie stained gels, comparing the amounts of S1 or FHS-S1 present in the supernatant and pellet relative to the amount present before centrifugation. The NH_4^+/Ca^{2+} ATPase activity of the resulting supernatants was also determined and compared with a zero-actin control to determine the amount of S1 or FHS-S1 bound to actin in the pellet (Chalovich and Eisenberg, 1982; Berger et al., 1989).

Fluorescence spectroscopy

Fluorescence emission spectra were collected with a Quantamaster fluorometer (Photon Technology International, South Brunswick, NJ). Samples of FHS-S1 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 with 1 μM FHS-S1 in the absence or presence of varying concentrations of F-actin at 25°C in 10 mM MOPS, 8 mM $MgCl_2$, and 25 mM EGTA, pH 7.1. Some experiments, as noted in the text, also included 2 mM ADP or 20 mM MgATP, and/or 150 mM KCl. F-actin concentrations varied from 5 to 10 μM for the rigor and MgADP experiments to 10–80 μM for the MgATP experiments. 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, TEMPO was added in increments of 10–20 mM up to 80 mM, and nitromethane, thallium acetate, and potassium iodide were added in increments of 5–10 mM up to 40 mM. Ionic strength was adjusted when necessary with KCl. The final concentrations of thallium acetate and potassium iodide added were limited by their contribution to the ionic strength of our solutions, and nitromethane by its solubility in 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 assess the accessibility of FHS at Lys-553 of myosin S1 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).

RESULTS

Labeling of myosin S1 with FHS

Skeletal muscle S1 was selectively labeled with FHS on the myosin heavy chain at a molar ratio of 0.84:1 (FHS/S1) as determined spectrophotometrically. Limited tryptic digestion of the myosin heavy chain indicated that the 50 kD subdomain was the sole site of fluorescent modification (Fig. 1), suggesting that Lys-553 was the predominate site of incorporation of FHS (Bertrand et al., 1995). Modification of Lys-553 resulted in small alterations in the actin-activated ATPase activity of FHS-S1 (Fig. 2). Unmodified S1 had a V_{max} of 11.8 s^{-1} and a K_m of 19 μM, while FHS-S1 had a V_{max} of 6.3 s^{-1} and a K_m of 26 μM, after adjustment for the fraction of unmodified S1 molecules (0.16). The relatively small decrease in V_{max} and lack of a significant change in the K_m value for FHS-S1 relative to unmodified S1 suggests that FHS-modified S1 is a functional myosin subfragment that can hydrolyze ATP in an actin-dependent manner, and interact normally with actin. Furthermore, FHS-S1 also binds as well to actin in a rigor or ADP-rigor complex in centrifuge pelleting assays as unmodified S1 (data not shown). The binding of FHS-S1 to actin during steady-state ATP hydrolysis (K_{eq} = 3.44 ± 0.36 × 10^3 M^{-1}) was only slightly lower than that of unmodified S1 (K_{eq} = 7.42 ± 0.41 × 10^3 M^{-1} (Berger et al., 1989)) as shown in Fig. 6 below.

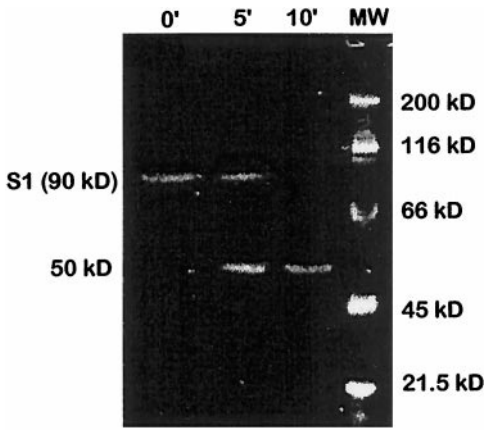

FIGURE 1 Fluorescent image of a limited tryptic digest of FHS-S1 (1:100 wt/wt ratio of trypsin/S1 at 25°C) run on a 6–12% gradient SDS-polyacrylamide gel. Lane 1: prior to addition of trypsin (0′). *Lanes 2* and *3:* at 5′ and 10′ after the addition of trypsin. *Lane 4:* fluorescent molecular weight standards (molecular weights (MW) are given at the right). The 50 kD fragment is selectively labeled by FHS.

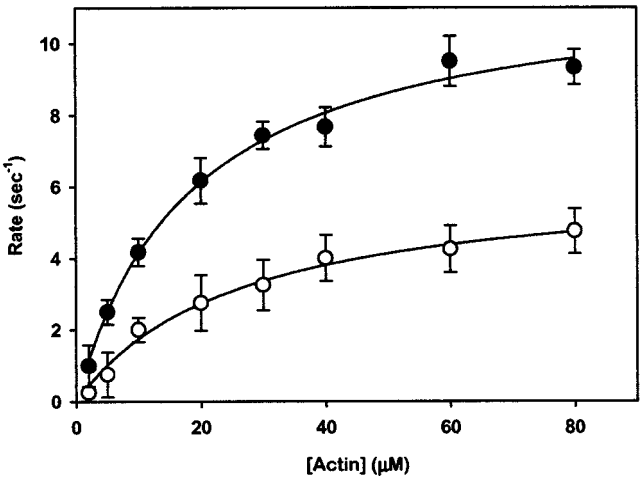

FIGURE 2 Actin-activated ATPase activities of unmodified (●) and FHS-labeled S1 (○). Data were fit assuming Michaelis-Menten kinetics. For unmodified S1 V_{max} = 11.8 s^{-1} and K_m = 19 μM. For FHS-S1 V_{max} = 6.3 s^{-1} and K_m = 26 μM, after correction for the fraction of unmodified S1 molecules (0.16).

Solvent quenching of the rigor acto-FHS-S1 complex

Fluorescence quenching experiments were carried out to examine the solvent accessibility of FHS at Lys-553 of skeletal muscle myosin S1 bound to actin in a rigor complex. Binding of actin did not appreciably change the fluorescence emission spectrum of FHS-S1 (data not shown). The fluorescence from FHS-S1 was effectively quenched by all four solvent quenchers used (Fig. 3), with TEMPO being the most efficient quencher used (K_{SV} = 22.5 ± 0.6 M^{-1}), followed by iodide (K_{SV} = 11.9 ± 0.3 M^{-1}), nitromethane (K_{SV} = 6.0 ± 0.5 M^{-1}), and thallium (K_{SV} = 6.0 ± 0.1 M^{-1}). When bound to actin in a rigor complex, FHS-S1 was quenched almost as effectively with TEMPO (K_{SV} = 20.7 ± 0.8 M^{-1}), nitromethane (K_{SV} = 5.5 ± 0.2 M^{-1}), and thallium (K_{SV} = 5.3 ± 0.3 M^{-1}) as in the absence of actin (Fig. 3). However, quenching by iodide was reduced almost twofold when FHS-S1 was bound to actin in a rigor complex (K_{SV} = 6.8 ± 0.4 M^{-1}) compared to FHS-S1

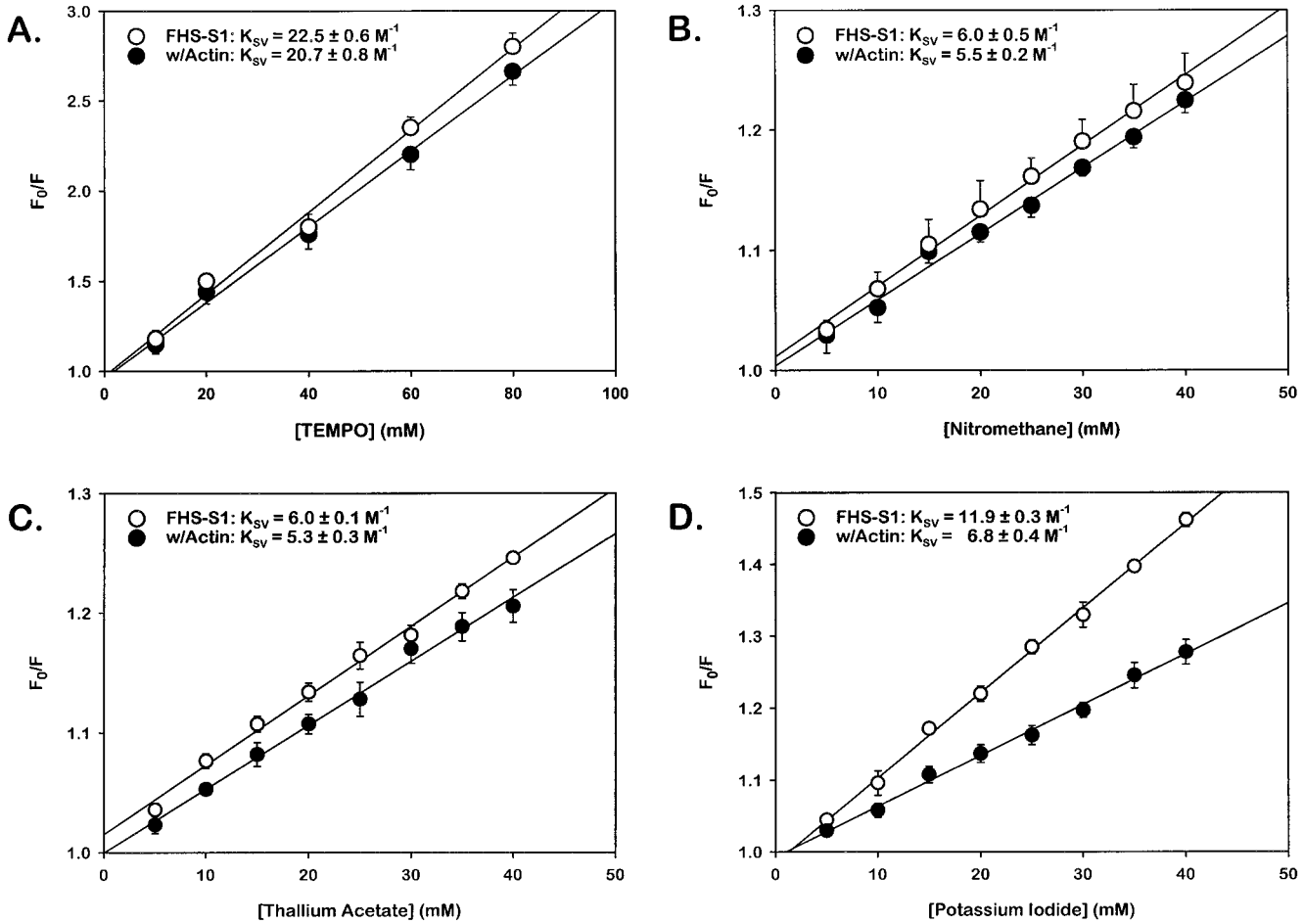

FIGURE 3 Stern-Volmer plots of 1 μM FHS-S1 alone (○) and in the presence of 5 μM actin (●) quenched by (*A*) TEMPO, (*B*) nitromethane, (*C*) thallium acetate, and (*D*) potassium iodide. K_{SV}, the Stern-Volmer quenching constant, was determined using Eq. 1. Values are given as the mean ± SE.

alone (Fig. 3). Together, these results suggest that the FHS probe at Lys-553 is not sterically protected from solvent access when myosin S1 is bound to actin in a rigor complex, but that actin may be providing an electrostatic shielding from the negatively charged iodide quencher. To further test this hypothesis, the iodide quenching experiments were repeated at a significantly higher salt concentration (Fig. 4). In the presence of 150 mM KCl, as expected, iodide was a less effective quencher of fluorescence from FHS-S1 alone (K_{SV} = 7.5 ± 0.2 M^{-1}). The protection offered from iodide quenching by actin binding, however, was virtually abolished (K_{SV} = 7.6 ± 0.7 M^{-1}), suggesting that the result observed at the lower salt concentration is indeed an electrostatic effect rather than a steric one.

Solvent quenching of the acto-FHS-S1-MgADP ternary complex

We also examined whether the structure of the acto-myosin interface at Lys-553 was altered by the binding of MgADP to the active site of myosin, which results in the formation of a strongly bound ternary complex. Addition of MgADP did not appreciably change the fluorescence emission spectrum of the rigor FHS-S1/actin complex (data not shown). Fluorescence quenching experiments using iodide as a solvent quencher were repeated as above for FHS-S1 and acto-FHS-S1 in the presence of 2 mM MgADP (Fig. 5). At low salt, the fluorescence from FHS-S1 was quenched effectively in the presence of 2 mM MgADP (K_{SV} = 12.2 ± 0.4 M^{-1}). Furthermore, as seen with the rigor acto-FHS-S1 complex, the degree of quenching decreased almost twofold when FHS-S1 was bound to actin in the presence of 2 mM MgADP (K_{SV} = 7.5 ± 0.4 M^{-1}). At higher salt concentrations (150 mM KCl) the results were again similar in the presence of 2 mM MgADP to those observed above in the absence of nucleotide. The overall degree of quenching decreased for FHS-S1 in the absence of actin (K_{SV} = 7.4 ± 0.4 M^{-1}), and was almost identical to that of FHS-S1 bound to actin (K_{SV} = 6.9 ± 0.3 M^{-1}). Thus, addition of MgADP to the rigor acto-S1 complex does not significantly alter the actin-binding interface of skeletal muscle myosin S1 at Lys-553.

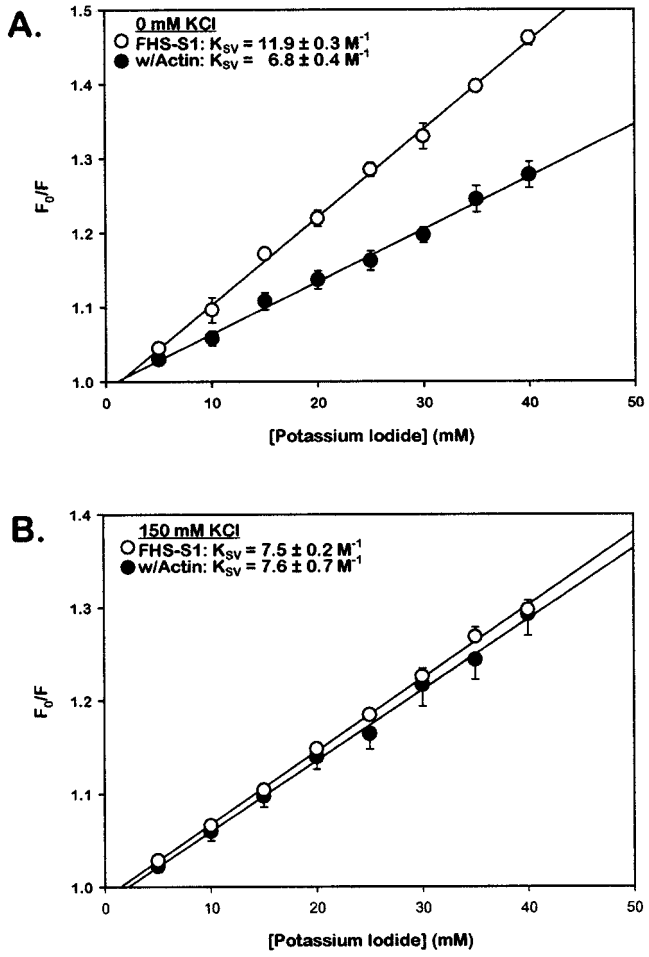

FIGURE 4 Stern-Volmer plots of 1 μM FHS-S1 alone (○) and in the presence of 5 μM actin (●) quenched by potassium iodide at (*A*) 0 mM KCl and (*B*) 150 mM KCl. K_{SV}, the Stern-Volmer quenching constant, was determined using Eq. 1. Values are given as the mean ± SE.

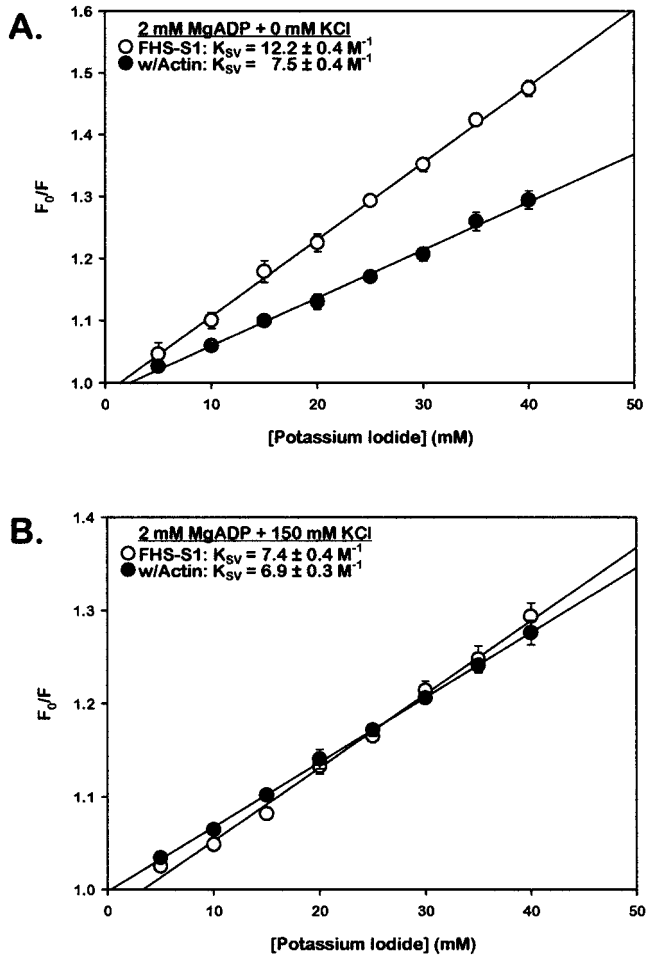

FIGURE 5 Stern-Volmer plots of 1 μM FHS-S1 + 2 mM MgADP (○) and in the presence of 5 μM actin + 2 mM MgADP (●) quenched by potassium iodide at (*A*) 0 mM KCl and (*B*) 150 mM KCl. K_{SV}, the Stern-Volmer quenching constant, was determined using Eq. 1. Values are given as the mean ± SE.

Solvent quenching during the steady-state hydrolysis of MgATP

Finally, we examined whether the structure of the acto-myosin interface at Lys-553 is different in the weakly bound states than in the strongly bound states. Addition of MgATP did not appreciably change the fluorescence emission spectrum of the rigor FHS-S1/actin complex (data not shown). Fluorescence quenching experiments using iodide as a solvent quencher were repeated as above with 1 μM FHS-S1 in the presence of 10−80 μM actin and 20 mM MgATP at low ionic strength. The fluorescence from FHS-S1 was just as effectively quenched by iodide in the presence of 20 mM MgATP at all actin concentrations tested as in the absence of actin, despite the fact the fraction of myosin heads bound to actin increased significantly from <5% at 10 μM actin to >20% at 80 μM actin (Fig. 6). Thus, the acto-myosin interface around Lys-553 of myosin is substantially altered in the weakly bound states during the steady-state hydrolysis of MgATP from that of the strongly bound states. These results suggest that the lower 50 kD subdomain of myosin containing Lys-553 only interacts with actin in the strongly bound states and not in the weakly bound states of the MgATPase cycle.

DISCUSSION

Summary of results

The accessibility of FHS, a fluorescent probe that specifically labels Lys-553 at the putative actin-binding interface of skeletal muscle myosin S1, was examined in the presence of a variety of neutral and charged solvent quenchers for FHS-S1 alone, complexed to actin in a strongly bound state (i.e., rigor or ADP-rigor), or complexed to actin in a weakly bound state (i.e., during steady-state ATP hydrolysis). FHS was equally accessible to neutral and positively charged

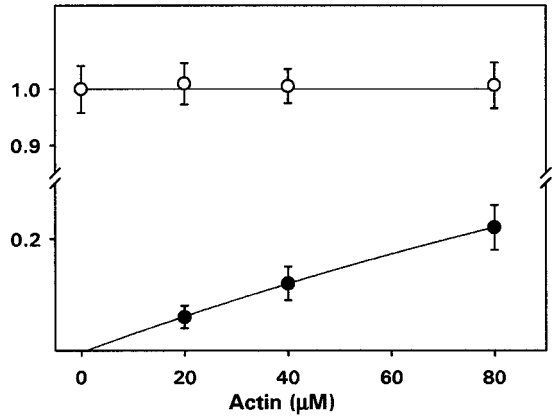

FIGURE 6 Relative Stern-Volmer constant calculated from potassium iodide quenching plots of 1 μM FHS-S1 (○) and fraction of 1 μM FHS-S1 bound to actin (●) as a function of actin concentration in the presence of 20 mM MgATP at low ionic strength (0 mM KCl). Stern-Volmer quenching constants were determined at each actin concentration using Eq. 1. The fraction of FHS-S1 molecules bound to actin at each actin concentration was determined from a centrifuge pelleting assay as described in Methods. Values are given as the mean ± SE.

solvent quenchers under all conditions studied, even in a rigor complex with actin. However, actin protects FHS from the negatively charged solvent quencher iodide in the strongly bound (i.e., the rigor and acto-FHS-S1-MgADP complexes), but not weakly bound (i.e., during steady-state ATP hydrolysis), states of the MgATPase cycle. Thus, the lower 50 kD subdomain of myosin containing Lys-553 appears to interact with actin differently in the weakly and strongly bound states. The protection offered to FHS by actin in the strongly bound states is abolished with the addition of relatively high concentrations of salt (150 mM KCl), suggesting that this effect is electrostatic rather than steric in nature, and that the helix containing Lys-553 in myosin binds near a negatively charged region of actin in the rigor and ADP-rigor complexes.

Fluorescent labeling of FHS-S1

Bertrand et al. (1995) originally reported that the myosin heavy chain can be extensively (0.9 mol FHS/mol S1) and specifically labeled by FHS. Furthermore, Lys-553 was the predominant site of modification by FHS, with only a relatively small amount (<15%) of additional modification at Lys-640 in the 50/20 kD loop (Bertrand et al., 1995). We have confirmed this result, demonstrating that only the 50 kD subdomain of myosin is fluorescently labeled by FHS at almost stoichiometric amounts (84%). While it is likely that our preparation also has been labeled to a small extent at Lys-640, the low level of incorporation at this site should not contribute greatly to our observed signal, of which at least 85% arises from the FHS-modified Lys-553 residues.

Modification of Lys-553 by FHS does slightly alter the enzymatic properties of myosin. Bertrand et al. (1995) reported that the K^+-EDTA ATPase activity of FHS-modified S1 is unaltered relative to unmodified S1, while the Ca^{2+}- and Mg^{2+}-ATPase activities are increased 150–200% over unmodified S1. Our preparation shows similar effects on the K^+-EDTA, Ca^{2+}-, and Mg^{2+}-ATPase activities of FHS-S1 (data not shown). Our actin-activated ATPase and actin-binding data are similar to those of Bertrand et al. (1995) as well, who reported a twofold decrease in V_{max} without a change in K_m, and no significant effect on the ability of FHS-S1 to bind to actin in a normal rigor complex. We also found no observable difference in the ability of FHS-S1 to bind to actin in the presence of MgADP, and only a minor difference in the binding of FHS-S1 to actin during steady-state ATP hydrolysis ($K_{eq} = 3.44 \pm 0.36 \times 10^3\ M^{-1}$), compared to that of unmodified S1 ($K_{eq} = 7.42 \pm 0.41 \times 10^3\ M^{-1}$ (Berger et al., 1989)).

Role of Lys-553 in actin binding

Three-dimensional reconstructions of cryoelectron micrographs of myosin subfragment 1 (S1) bound to F-actin have been used to "dock" the atomic structures of S1 and actin together in a rigor complex (Rayment et al., 1993b; Milligan, 1996; Mendelson and Morris, 1997). The resulting models postulate that myosin binds to actin in a sequential, multi-step process, in which myosin first binds to actin in a weakly bound, rapid equilibrium state, followed by the formation of a strongly bound, stereospecific complex. Lys-553 is located in the middle of the second α-helix (residues Asp-547 to His-558) in the helix-loop-helix motif of the lower 50 kD subdomain of myosin that is thought to be one of these putative actin-binding regions (Rayment et al., 1993b). However, it is unclear whether this site in myosin is involved in binding to actin in the weakly bound state, the strongly bound state, or both. Bertrand et al. (1995) demonstrated that labeling Lys-553 with FHS could be specifically blocked when myosin was bound to actin in a rigor complex, suggesting that this residue forms part of the actin-binding interface. However, it is also possible that actin induces local conformational changes in myosin that make Lys-553 less accessible to or less reactive with FHS. Onishi et al. (1995) demonstrated that mutating two hydrophobic amino acids in the same α-helix as Lys-553 to more polar residues (W546S and F547H) results in greatly depressed actin-activated ATPase activities. More recently, we have demonstrated that the fluorescence emission from tryptophan 546 in smooth muscle myosin undergoes a substantial blue-shift upon actin binding in the rigor and ADP-rigor complexes (Yengo et al., 1998; 1999). Taken together, these results all support a model in which the α-helix containing Lys-553 plays an active role in binding to actin in the strongly bound states (i.e., the ADP and rigor states). Our current results also support such a model, since the FHS

probe at Lys-553 is close enough to actin to be protected from solvent quenching by potassium iodide in the strongly bound rigor and ADP-rigor states.

It is possible that the α-helix in myosin containing Lys-553 plays an active role in binding to actin in the weakly bound states as well (i.e., the ATP and ADP · P_i states). The weakly bound state is believed to involve a primarily ionic interaction between the positively charged, flexible surface loop that links the 50 kD and 20 kD tryptic fragments of the myosin heavy chain and the negatively charged N-terminus of actin. Evidence for the interaction of the 50/20 kD junction in myosin with the N-terminus of actin in the weakly bound state includes the protection of this loop from proteolytic cleavage by actin (Mornet et al., 1979), and alterations in this loop impact the actin-activated ATPase and actin-binding activities of myosin (Uyeda et al., 1994; Rovner et al., 1995). However, we find that during steady-state ATP hydrolysis, when myosin is predominantly in the weakly bound state, that actin offers virtually no protection from iodide quenching, even when the actin concentrations are sufficiently high (80 μM) to have a significant fraction of the FHS-S1 molecules bound to actin (>20%). *Thus, our results demonstrate that it is unlikely that the lower 50 kD subdomain of myosin containing Lys-553 interacts with actin in the weakly bound states.* At the very least, such an interaction must be substantially different in the weakly and strongly bound states to account for the differences in the accessibility of the FHS probe at Lys-553 under the two different sets of experimental conditions. *This is the first direct evidence for a difference in the way that the lower 50 kD subdomain interacts with actin in the weakly and strongly bound states*. Thus, incorporation of the lower 50 kD subdomain of myosin may be the first step in the formation of the strongly bound complex between actin and myosin.

In the strongly bound states, why is the FHS group covalently bound to Lys-553 only protected from solvent quenching by actin when iodide is used as the quencher, and not nitromethane, TEMPO, or thallium? The six-carbon spacer arm between the reactive succinimidyl and fluorescent fluorescein groups in FHS may provide enough distance and flexibility to keep the bulky fluorophore out of the actual interface between actin and myosin. This would explain the lack of an effect by FHS labeling on rigor binding and the K_m for actin-activated ATPase activity. Furthermore, if the fluorescein group is indeed at the edge of the actin-binding interface of myosin, the predominantly negatively charged surface of actin would be expected to protect FHS from quenching by the negatively charged iodide ion by electrostatic repulsion. However, the neutral quenchers nitromethane and TEMPO would not be sterically or electrostatically shielded from FHS by actin, and thus should effectively quench the fluorescein fluorescence even in the ADP-rigor and rigor complexes. If electrostatic shielding is indeed the mechanism by which actin protects the FHS probe on myosin, it is curious that actin does not enhance quenching by the positively charged thallium ion. One possible explanation is that positive charges on myosin around the FHS-probe repel the thallium ion in both the free and actin-bound states of myosin, and would also act to enhance the quenching efficiency of iodide for FHS-S1 alone, particularly at low salt concentrations as observed in our data.

It is important to note that no difference was observed in the ability of iodide to quench FHS-S1 in either the ADP-rigor or rigor complexes, suggesting that the interaction between the helix containing Lys-553 in myosin and actin is unaltered by ADP release. Whittaker et al. (1995), using reconstructions of cryo-EM images, found a small but statistically significant difference in the acto-myosin interface of smooth muscle myosin upon ADP release. Thus, rearrangements in the acto-myosin interface are likely to occur elsewhere in myosin and/or in actin. We have recently demonstrated using intrinsic tryptophan fluorescence that the myopathy loop (residues Arg-405 to Lys-415 in the skeletal muscle myosin sequence) in the upper 50 kD subdomain of smooth muscle myosin alters its conformation upon ADP release when complexed with actin, while the lower 50 kD subdomain containing Lys-553 is unaffected by ADP release (Yengo et al., 1999).

Future directions

Given the large negative surface potential of actin it is difficult to speculate which regions of actin the fluorescein probe may be near in the rigor complex. Fluorescence resonance energy transfer experiments (FRET) between FHS at Lys-553 in myosin and donor probes on actin (e.g., IAEDANS at Cys-374 or dansyl at Gln-41) may be useful in properly orienting the actin and myosin molecules with each other in the rigor complex. FHS-labeled S1 has already been used as acceptor for determining intermolecular distances by FRET within the myosin head, using IAEDANS conjugated to Cys-177 of the essential light chain as the donor (Smyczynski and Kasprzak, 1997). Transient kinetic analysis of the fluorescence emission in the presence of potassium iodide following rapid mixing of FHS-S1 and actin may also help address questions about the order in which different subdomains of myosin bind to actin in forming the strongly bound complex.

The sensitivity of FHS-labeled myosin heads to iodide quenching as a function of actin binding reported in this work may be applicable to determining the fraction of actin-bound cross-bridges in the myofilament lattice, assuming that Lys-553 can still be specifically modified in myosin under these conditions. In the accompanying paper (Cooper et al., 2000), we demonstrate that myosin can be specifically labeled at Lys-553 in skeletal muscle myofibrils, and the FHS probe retains it sensitivity to iodide quenching in the relaxed state, but not in the rigor state, at

low ionic strength. This technique should have direct applications for the real-time determination of the fraction of actin-bound cross-bridges within the myofilament lattice under a variety of mechanical conditions, and help address critical questions about chemomechanical coupling in muscle contraction.

CONCLUSIONS

We found that actin protects the FHS probe at Lys-553 of the myosin heavy chain from solvent quenching by potassium iodide in the strongly bound (i.e., the rigor and acto-FHS-S1-MgADP complexes), but not weakly bound (i.e., during steady-state ATP hydrolysis), states of the MgATPase cycle. Thus, it appears that the lower 50 kD subdomain of myosin containing Lys-553 interacts with actin differentially in the strongly and weakly bound states. Such a dynamic structural change at the acto-myosin interface may provide at least part of the mechanism responsible for the large affinity change between actin and myosin at this critical transition in the MgATPase cycle.

The authors thank Alan DeGeorge, Beth Schofield, and Chris Yengo 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.). J.J.M. was supported in part by a fellowship from the HeliX undergraduate research program at the University of Vermont.

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