Antibody and Peptide Probes of Interactions between the SH_1–SH_2 Region of Myosin Subfragment 1 and Actin's N-Terminus†

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ABSTRACT: The negatively charged residues in the N-terminus of actin and the 697–707 region on myosin subfragment 1 (S-1), containing the reactive cysteines SH_1 and SH_2, are known to be important for actin-activated myosin ATPase activity. The relationship between these two sites was first examined by monitoring the rates of SH_1 and SH_2 modification with *N*-ethylmaleimide in the presence of actin and, secondly, by testing for direct binding of SH_1 peptides to the N-terminal segment on actin. While actin alone protected SH_1 from *N*-ethylmaleimide modification, this effect was abolished by an antibody against the seven N-terminal amino acids on actin, $F_{ab}(1–7)$, and was greatly reduced when the charge of acidic residues at actin's N-terminus was altered by carbodiimide coupling of ethylenediamine. Neither $F_{ab}(1–7)$ nor ethylenediamine treatment reversed the effect of F-actin on SH_2 reactivity in SH_1-modified S-1. These results show a communication between the SH_1 region on S-1 and actin's N-terminus in the acto–S-1 complex. To test whether such a communication involves the binding of the SH_1 site on S-1 to the N-terminal segment of actin, the SH_1 peptide IRICRKG-NH_2(4+) was used. Cosedimentation experiments revealed the binding of three to six peptides per actin monomer. Peptide binding to actin was affected slightly, if at all, by $F_{ab}(1–7)$. The antibody also did not change the polymerization of G-actin by the peptides. The peptides caused a small reduction in the binding of S-1 to actin and did not change the binding of $F_{ab}(1–7)$. Comparison with the peptide IRICRKG(3+) and the scrambled peptide RICIRGK(3+) showed that charge is the dominant factor in binding of SH_1 peptides to actin in solution, as in skinned fibers, and suggested limitations in the use of these peptides as probes mimicking the SH_1 region on S-1. Taken together, these experiments suggest that the N-terminus of actin interacts indirectly with the SH_1 site on intact S-1.

It is generally accepted that muscle contraction results from cyclic interactions between actin and myosin filaments. Coupled to adenosine 5′-triphosphate (ATP)[1] hydrolysis, these interactions result in generation of muscular force and relative sliding of the contractile filaments. Actin activates myosin ATPase activity by accelerating product release [for reviews, see Taylor (1979), Adelstein and Eisenberg (1980), and Eisenberg and Hill (1985)], and product release is closely linked to force generation [for reviews, see Hibberd and Trentham (1986) and Homsher and Millar (1990)]. However, the structural basis of these processes is yet to be elucidated at the molecular level.

Consequently, there is much interest in defining regions on actin and myosin which are important for the actomyosin ATPase activity. The N-terminal 1–7 residues of actin bind S-1 and play an important role in actomyosin·nucleotide interactions (Mornet et al., 1981; Sutoh, 1982; Miller et al., 1987; DasGupta & Reisler, 1989, 1991). The acidic residues in this segment of actin appear to be required for the activation of the myosin ATPase. Activation of myosin ATPase is greatly reduced if the negative charges at actin's N-terminus are altered either by chemical modification (Bertrand et al., 1989; Cheung and Reisler, unpublished results) or by site-directed mutagenesis (Sutoh et al., 1991; Cook et al., 1992). Likewise, blocking the N-terminus of actin by anti-actin $F_{ab}(1–7)$ sharply reduces actin-activated S-1 ATPase activity (Myint et al., 1991; DasGupta & Reisler, 1989, 1992).

In myosin, specific chemical modifications of the reactive thiols SH_1 and SH_2 (Cys-707 and -697, respectively; Gallagher & Elzinga, 1980) dramatically alter the ATPase activities of both myosin (Sekine & Kielley, 1964; Yamaguchi & Sekine, 1966) and actomyosin (Silverman et al., 1972; Root & Reisler, 1992). When these two thiols are chemically cross-linked, the binding affinity of S-1 for actin is greatly reduced in solution (Reisler et al., 1974; Chalovich et al., 1983) and in permeabilized muscle cells (Chaen et al., 1986; Barnett et al., 1992). These results suggest that the highly conserved SH_1–SH_2 region may be centrally involved in signal transmission between the actin and nucleotide binding sites on S-1 (Botts et al., 1984, 1989). However, since SH_1 is probably located 4–6 nm from both the actin and nucleotide binding sites on S-1 (Tokunaga et al., 1987; Botts et al., 1989) and blocking of this

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[1] Abbreviations: S-1, myosin subfragment 1; SH_1 and SH_2, cysteine-707 and -697 of rabbit skeletal myosin, respectively; SH_1-blocked S-1, S-1 premodified at cysteine-707 by NEM; SH_1 peptides, heptapeptides containing the 704–710 sequence (IRICRKG) of rabbit skeletal myosin or single amino acid substitutions; $F_{ab}(1–7)$, affinity-purified F_{ab} fragment of polyclonal antibodies directed against the N-terminal 1–7 sequence (acetyl-DEDETTAY) of α-actin; ED-actin, actin labeled with ethylenediamine (ED); MBS-actin, *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester modified actin; pyrene-labeled actin, actin labeled at cysteine-374 by *N*-(1-pyrenyl)iodoacetamide; DTT, dithiothreitol; ATP, adenosine 5′-triphosphate; EDC, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide; NEM, *N*-ethylmaleimide; MOPS, 3-(*N*-morpholino)propanesulfonic acid; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; SDS, sodium dodecyl sulfate; NMR, nuclear magnetic resonance spectroscopy.

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cysteine on S-1 with biotin–avidin did not abolish actin binding or actin activation of S-1 ATPase activity (Yamamoto & Sekine, 1987), binding of SH_1 itself to actin appeared unlikely.

More recently, synthetic peptides containing sequences derived from the SH_1–SH_2 region of S-1 have been used for probing the role of this region in actomyosin interactions. SH_1 peptides bind to actin and inhibit both actomyosin ATPase activity in solution and force generation in muscle fibers (Suzuki et al., 1987, 1990; Keane et al., 1990; Chase et al., 1991). In addition, the SH_1 heptapeptide (containing sequence 704–710 of rabbit skeletal S-1) induces polymerization of G-actin as does S-1 (Eto et al., 1991). The effects of cationic SH_1 peptides on actomyosin ATPase activity (Suzuki et al., 1990; Keane et al., 1990) and an observation that some ^{1}H-NMR resonances from actin peptide 1–44 were quenched by S-1 that was spin-labeled at SH_1 (Keane et al., 1990) prompted speculation about the possible binding of the SH_1 region to the negatively charged residues (1–4) in the N-terminus of actin. However, the specificity of peptide interactions with actin, at least in the fiber, has been cast in doubt by the finding that their inhibitory effects on force generation and ATPase activity in muscle fibers were charge- and not sequence-dependent (Chase et al., 1991).

These observations prompted the examination in the present work of the interrelationship between the SH_1 region of S-1 and the N-terminal segment on actin. To test for the interaction between these sites in acto–S-1 complexes, the modifications of SH_1 and SH_2 by *N*-ethylmaleimide were monitored in the presence of (i) actin, (ii) actin complexed with the N-terminal antibody F_{ab}(1–7), and (iii) actin with its N-terminal acidic residues modified by carbodiimide coupling of ethylenediamine. The results of these experiments showed that perturbation of the N-terminal segment of actin affected the SH_1–SH_2 region of myosin and thus suggested either direct or indirect interactions between the respective actin and S-1 sites. It appeared that the simplest way to distinguish between these possibilities was to examine the binding to F-actin of three peptides: IRICRKG-NH_2(4+), IRICRKG(3+), and RICIRGK (scrambled 3+ peptide). According to solution studies (Suzuki et al., 1990), but not the fiber work (Chase et al., 1991), IRICRKG(4+) could mimic the binding of the SH_1 region on S-1 to actin. We found three to six peptide molecules bound per actin subunit; peptide binding to actin neither affected nor was affected by F_{ab}(1–7). Peptide binding to G-actin was evidenced by induction of actin polymerization by the peptides; as with binding to F-actin, polymerization of G-actin by these peptides was unchanged by F_{ab}(1–7). Although these results favor the interpretation of indirect interaction between the SH_1 site on S-1 and the N-terminus on actin, they also point to the limitations of these peptides as specific probes mimicking the SH_1 region on S-1.

MATERIALS AND METHODS

Reagents. *N*-Ethylmaleimide, ATP, ethylenediamine, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide, TLCK-treated α-chymotrypsin, phenylmethanesulfonyl fluoride, and dithiothreitol were purchased from Sigma Chemical Co. (St. Louis, MO). *N*-(1-Pyrenyl)iodoacetamide was purchased from Molecular Probes (Eugene, OR). All other reagents were analytical grade and were obtained from Fisher Scientific Co.

Peptide Synthesis. The synthetic peptides IRICRKG-NH_2-(4+), IRICRKG(3+), and RICIRGK (scrambled 3+ peptide) and a Thr-substituted SH_1 peptide, IRITRKG(3+), were synthesized by the Howard Hughes Chemical Synthesis Facility, University of Washington (Seattle, WA), and were purified and analyzed as described previously (Chase et al., 1991). The peptides were solubilized in 0.1 mM KCl, 5 mM DTT, and 20 mM MOPS, pH 7.0, at a stock concentration of 5 mM, and aliquots were stored at −20 °C.

Proteins. Actin and myosin were prepared from rabbit skeletal muscle according to the methods of Spudich and Watt (1971) and Godfrey and Harrington (1970), respectively. Myosin subfragment 1 (S-1) was prepared by α-chymotryptic cleavage of myosin according to the method of Weeds and Pope (1977). Antibodies against the 1–7 sequence of α-actin and their F_{ab} fragments were prepared as described by Miller et al. (1987). Protein concentrations were determined spectrophotometrically at 280 nm by using $E^{1\%} = 11.0$ cm^{-1} for actin, 7.5 cm^{-1} for S-1, and 16.0 cm^{-1} for F_{ab}. The molecular masses of the proteins are as follows: S-1, 110 kDa; S-1, 110 kDa; actin, 42 kDa; F_{ab}, 50 kDa.

Pyrene Modification of Actin. Pyrene-labeled actin was prepared according to the method of Cooper et al. (1983). The concentration of labeled actin was determined by Bradford assay (Bradford, 1976). The extent of pyrene labeling was determined by using a molar extinction coefficient of $E_{344} = 22\,000$ M^{-1} cm^{-1} for the pyrene–actin complex (Kouyama & Mihashi, 1981). The labeling stoichiometry was between 0.5 and 0.9 pyrene/actin.

Ethylenediamine Modification of Actin. Ethylenediamine-modified actin (ED-actin) was prepared essentially as described by Adams et al. (1990) except that the reaction was carried out in 0.1 M KCl, 2 mM $MgCl_2$, and 10 mM HEPES, pH 7.0, using 5 mM ED and 7.5 mM EDC. The coupling was terminated with 10 mM DTT. The extent of actin modification was monitored by measuring activation of S-1 ATPase activity which was inhibited by 85–90% following modification. After modification, the ED-actin was exhaustively dialyzed overnight against 30 mM KCl and 25 mM Tris, pH 7.6, and used within 24 h.

N-Ethylmaleimide (NEM) Modification of SH_1 and SH_2 in S-1. NEM modification of S-1 was carried out in the absence and presence of either unmodified actin or ED-actin. In some experiments with unmodified actin, either F_{ab}(1–7) or a Thr-substituted SH_1 peptide, IRITRKG, was also added. In general, S-1 was modified with NEM (2–4-fold molar excess over S-1) in 30 mM KCl and 25 mM Tris, pH 7.6, at 0 °C. During the reaction, aliquots were transferred at the indicated time points to test tubes containing 1 mM DTT. These samples were then assayed for the K^+-EDTA-ATPase and Ca^{2+}-ATPase activities of S-1 (Reisler, 1980).

To prepare SH_1-blocked S-1, S-1 (20 μM) was reacted with 40 μM NEM for 15 min, at which time the Ca^{2+}-ATPase activity of S-1 was maximally activated (5-fold) and more than 95% of the K^+-EDTA-ATPase activity was lost, indicating nearly complete labeling of SH_1 (Sekine & Kielley, 1964; Reisler et al., 1974). The SH_1-blocked S-1 was exhaustively dialyzed against 30 mM KCl and 25 mM Tris, pH 7.6, overnight and then subjected to a second NEM modification to label the SH_2 at a 4 to 1 molar ratio of NEM to S-1. The extent of SH_2 modification was monitored by measuring the decrease in the Ca^{2+}-ATPase activity (Yamaguchi & Sekine, 1966).

ATPase Assays. K^+-EDTA and Ca^{2+}-ATPase activities of S-1 were measured at 37 °C according to the methods of Kielley et al. (1956) and Kielley and Bradley (1956). The actin-activated ATPase activity of S-1 was measured at 25 °C essentially as described by Reisler (1980). The assay

mixture contained S-1 (1.0 μM) and either unmodified actin (25.0 μM) or ED-actin (25.0 μM) in 30 mM KCl, 2 mM $MgCl_2$, 2 mM ATP, and 25 mM Tris, pH 7.6. When peptides were included in the measurements, the assay mixture contained S-1 (3.0 μM), actin (3.0 μM), and one of the three peptides (up to 1.2 mM) in 10 mM KCl, 3 mM $MgCl_2$, 3 mM ATP, 5 mM DTT, and 10 mM imidazole, pH 7.0. Liberated phosphate was measured colorimetrically (Fiske & Subbarow, 1925).

Cosedimentation Binding Measurements. The effects of peptides on the binding of S-1 or $F_{ab}(1–7)$ to actin were monitored essentially according to the procedures of Suzuki et al. (1987). In short, S-1 (4.0 μM) or $F_{ab}(1–7)$ (3.0 μM) was incubated with F-actin (5.0 μM) in the absence and presence of one of the peptides (1.2 mM) for 30 min at 22 °C in 0.1 M KCl, 5 mM DTT, and 20 mM MOPS, pH 7.0. The mixtures were then pelleted in a Beckman airfuge for 30 min at 22 °C to separate the bound and unbound peptides, S-1, and $F_{ab}(1–7)$. Under the experimental conditions employed, little or no peptide or S-1 was pelleted in the absence of actin.

SDS–Polyacrylamide Gel Electrophoresis and Densitometric Analysis. The supernatants and pellets, containing free proteins and peptides and those bound to actin, respectively, were denatured and subjected to Tricine-buffered SDS–polyacrylamide gel electrophoresis using two-layered gels consisting of 10% (upper layer) and 20% (lower layer) acrylamide. This system offered better resolution of small molecular weight peptides than the commonly used glycine-buffered system (Schägger & von Jagow, 1987). Coomassie blue stained peptide and protein bands were scanned with a Biomed Instruments SLR 2D/1D soft laser scanning densitometer interfaced to a DTK computer. The molar ratios of peptides bound per actin monomer in F-actin were calculated on the basis of their stain ratios determined from the linear stain intensity/quantity curves of known amounts of peptides and actin run on the same gel.

Polymerization of G-Actin by Peptides. The polymerization of G-actin by peptides in the absence and presence of $F_{ab}(1–7)$ was monitored in a SPEX FLUOROLOG spectrophotometer by measuring the increase in the fluorescence intensity of pyrene-labeled G-actin (Cooper et al., 1983; Miller et al., 1987). In short, G-actin (4.0 μM, 50% pyrene-labeled) in 0.2 mM $CaCl_2$, 0.2 mM ATP, 0.5 mM β-mercaptoethanol, and 5 mM Tris, pH 7.6, was polymerized at 25 °C by a 0.2 mM aliquot of one of the peptides in the absence and presence of F_{ab} (8.0 μM). The excitation and emission wavelengths were 368 and 408 nm, respectively. Fluorescence intensities are shown in arbitrary units. The effects of peptides and $F_{ab}(1–7)$ were assessed by comparing both the rates and final levels of actin polymerization. The initial rates of actin polymerization ($\Delta F/\Delta t \Delta F_{max}$) were corrected for the final levels of total actin polymerized.

RESULTS

Effects of Blocking the N-Terminal Segment of Actin by $F_{ab}(1–7)$ on the Reactivities of SH_1 and SH_2 on S-1. Figure 1 shows the time course of NEM modification of S-1 as monitored by assaying the Ca^{2+}-ATPase (Figure 1A) and K^+-EDTA-ATPase activities (Figure 1B) of S-1. It is well established that modifications of the SH_1 group (Cys-707) result in marked elevation of the Ca^{2+}-ATPase activity of S-1 and the accompanying loss of its K^+-EDTA-ATPase activity (Sekine & Kielley, 1964; Yamaguchi & Sekine, 1966; Reisler et al., 1974). These characteristic changes in ATPase activities of S-1 upon modification of SH_1 groups are seen also in Figure 1. In the presence of actin, the reactivity of the SH_1 group is greatly reduced (Duke et al., 1976). Consequently, the Ca^{2+}-ATPase and K^+-EDTA-ATPase activities of S-1 change very little when the reaction is carried out on acto–S-1 complexes.

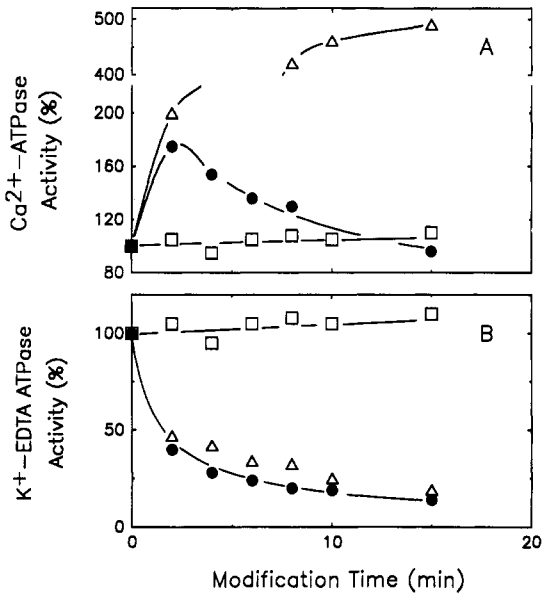

FIGURE 1: Time course of NEM modification of S-1 in the absence and presence of actin and $F_{ab}(1–7)$. The modification reactions were monitored by measurements of (A) Ca^{2+}-ATPase and (B) K^+-EDTA-ATPase activities of S-1. In these modifications, 3.0 μM S-1 was reacted with 6.0 μM NEM in the absence (△) and presence of 3.0 μM actin (□) or 3.0 μM actin and 10.0 μM $F_{ab}(1–7)$ (●). The modification and assay procedures were as described under Materials and Methods.

However, when S-1 is modified by NEM in the presence of both actin and $F_{ab}(1–7)$, the initial increase of Ca^{2+}-ATPase activity and rapid loss of the K^+-EDTA-ATPase activity suggest that in this case SH_1 is not protected from labeling (Figure 1A,B). Furthermore, a subsequent decrease in the Ca^{2+}-ATPase activity of S-1 (Figure 1A) indicates that SH_2 is also labeled by NEM (Sekine & Kielley, 1964; Yamaguchi & Sekine, 1966). As evidenced by pelleting of acto–S-1 complexes and analysis of these pellets on SDS–PAGE, between 80 and 85% of S-1 is bound to actin in the presence of $F_{ab}(1–7)$ under the conditions employed in this work [data not shown; see also Miller et al. (1987)]. Thus, the results show that blocking the actin's N-terminus by $F_{ab}(1–7)$ changes the reactivities of SH_1 and SH_2 on S-1 that is bound to actin.

Modification of SH_1 Groups on S-1 in the Presence of Actin Modified with Ethylenediamine. The acidic residues in actin's N-terminus can be modified with a high degree of specificity using an EDC-catalyzed reaction (Bertrand et al., 1989; Elzinga, 1986). Modification with ethylenediamine (ED) via EDC introduces positive charges into the N-terminus of actin (Elzinga, 1986) and inhibits up to 90% of the ability of actin to activate S-1 ATPase activity without reducing greatly the rigor acto–S-1 binding (Adams et al., 1990). When S-1 is modified by NEM in the presence of ED-actin, the changes in the Ca^{2+}-ATPase (Figure 2) and K^+-EDTA-ATPase (not shown) activities of S-1 indicate that the reactivity of SH_1 is increased to an intermediate level between that of free S-1 and that of S-1 in the presence of unmodified actin. Unlike actin with $F_{ab}(1–7)$ bound to it, ED-modified actin does not accelerate the labeling of SH_2 on S-1. These results show that changes in the electrostatic charge at the N-terminus of actin affect the reactivity of SH_1 but not the reactivity of SH_2 in S-1.

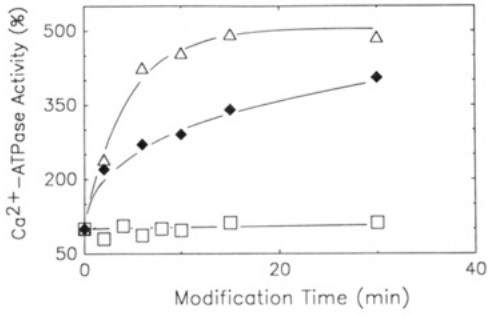

FIGURE 2: Effect of ethylenediamine modification of actin on NEM labeling of SH_1 and SH_2 groups on S-1. The reactions were monitored by measurements of the Ca^{2+}-ATPase activity of S-1. In these reactions, 5.0 μM S-1 was modified by 10.0 μM NEM in the absence (△) and presence of 7.0 μM actin (□) or 7.0 μM ED-actin (♦).

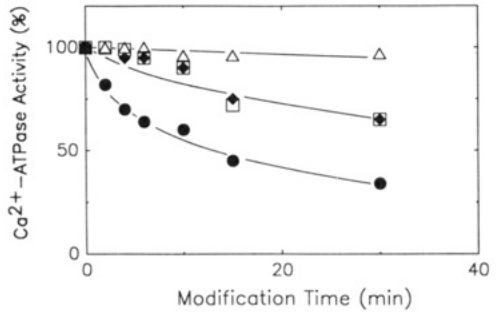

FIGURE 3: Time course of NEM modification of the SH_2 group in SH_1-blocked S-1 as monitored by measurements of the Ca^{2+}-ATPase activity of S-1. SH_1-blocked S-1 (3.0 μM) was modified by 12.0 μM NEM either alone (△) or in the presence of unmodified actin (6.0 μM, □), ED-actin (6.0 μM, ♦), or actin + F_{ab}(1–7) (6 and 14.0 μM, respectively, ●). SH_1-blocked S-1 was prepared as described under Materials and Methods.

The SH_2 of S-1 and the N-Terminus of Actin. Actin enhances the reactivity of SH_2 on myosin in which SH_1 has been preblocked (Kameyama & Sekine, 1973). Modification of SH_2 in such myosin is monitored by following the corresponding decrease in the Ca^{2+}-ATPase activity, which was elevated by the previous reaction of SH_1 groups (Yamaguchi & Sekine, 1966; Figures 1 and 2). As shown in Figure 3, actin and ED-actin accelerate the rate of SH_2 modification in SH_1-blocked S-1 to a similar extent. Using F_{ab}(1–7) to block the N-terminus of actin further increased the reactivity of SH_2 (Figure 3), as might be expected from the results shown in Figure 1. Under our experimental conditions, about 70% of SH_1-blocked S-1 is bound to actin in the presence of F_{ab}(1–7) (data not shown).

Taken together, the above results demonstrate that the reactivities of SH_1 and SH_2 on S-1 bound to actin are affected strongly by manipulating the structure and charge of the N-terminal region of actin. SH_1 appears to be more sensitive to electrostatic changes at actin's N-terminus and the binding of F_{ab}(1–7) than SH_2. The greater sensitivity of SH_1 may be related to its location on S-1, the adjacent sequence, and perhaps its proximity or even binding to the N-terminus of actin in the acto–S-1 complex. In order to assess that latter possibility, the interaction of SH_1 peptides with actin in solution was examined by using several approaches.

Effect of SH_1 Peptides on Acto–S-1 Interactions. Three SH_1 peptides, IRICRKG-NH_2(4+), IRICRKG(3+), and RICIRGK(3+) which were scrutinized in previous work on permeabilized muscle cells (Chase et al., 1991), were also tested here for binding to F-actin, for effects on acto–S-1 ATPase activity and binding of S-1 or F_{ab}(1–7) to F-actin, and for the ability to polymerize G-actin. A fourth peptide, IRITRKG(3+), was tested for its ability to reverse F-actin's protection of SH_1 on S-1 from modification by NEM. These experiments were designed to probe the possible binding of the IRICRKG-NH_2(4+) peptide to the N-terminal segment of actin, and also, by using the additional peptides, to assess the specificity of the peptide–actin interactions.

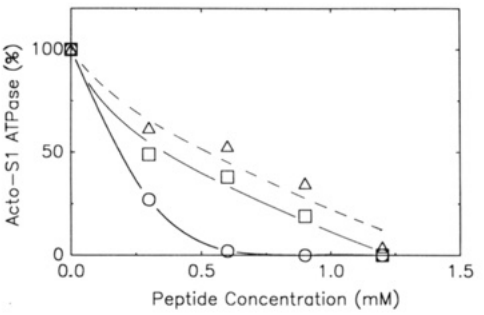

FIGURE 4: Inhibition of acto–S-1 ATPase activity by the peptides. ATPase activities were measured in the presence of IRICRKG-NH_2 (○), IRICRKG (□), and RICIRGK (△) in 10 mM KCl, 5 mM DTT, 3 mM $MgCl_2$, 3 mM ATP, and 10 mM imidazole, pH 7.0, at 25 °C. Actin and S-1 concentrations were 3.0 μM each, and peptide concentrations varied as indicated.

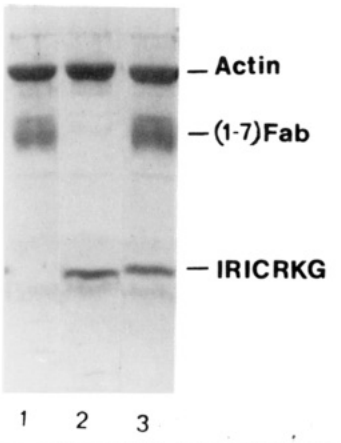

FIGURE 5: Representative SDS–polyacrylamide gels showing the binding of F_{ab}(1–7) and the IRICRKG peptide to actin. F-Actin (5.0 μM) was pelleted in the airfuge in the presence of 3.0 μM F_{ab} (lane 1), or 1.2 mM peptide (lane 2), or both (lane 3). The pellets containing the bound F_{ab} and peptide, as shown here, were analyzed as described under Materials and Methods. The results of such experiments are summarized in Table II.

As shown in Figure 4, the acto–S-1 ATPase activity was inhibited by all three peptides. The range of peptide concentrations at which half-maximal inhibition occurred ($K_{1/2}$) was similar to that reported by Suzuki et al. (1990) for the SH_1 heptapeptide. In agreement with Chase et al. (1991), the actual values of $K_{1/2}$—0.13 mM for IRICRKG-NH_2-(4+), 0.35 mM for IRICRKG(3+), and 0.50 mM for RICIRGK(3+)—show that, as in muscle fibers, peptide inhibition of acto–S-1 ATPase activity in solution depends more on the net charge than on the sequence of the peptide.

The binding of peptides and S-1 to actin was measured by pelleting these complexes and subsequent SDS–PAGE analysis of the protein and peptide bands found in the pellets (Figure 5). Table I shows that all three peptides (1.2 mM) inhibited somewhat the binding of S-1 to actin and, within the resolution of these measurements, probably to a similar extent. Reciprocally, S-1 appears to inhibit the binding of the two 3+ peptides to actin and less so, if at all, the binding of the 4+ peptide (Table I). The slight inhibition of S-1 binding to actin by IRICRKG and IRICRKG-NH_2 is consistent with the results of Suzuki et al. (1987). Yet the specificity of this

Table I: Binding of S-1 and Peptides to Actin in the Presence of Each Other[a]

peptide (1.2 mM)	% binding S-1	% binding peptide
IRICRKG-NH_2	90 ± 6	100 ● 10
IRICRKG	85 ± 6	84 ± 5
RICIRGK	90 ± 1	82 ± 5

[a] The binding of S-1 and peptides to actin was measured in 0.1 M KCl, 5 mM DTT, and 20 mM MOPS, pH 7.0, at 22 °C. Actin, S-1, and peptide concentrations were 5.0 μM, 4.0 μM, and 1.2 mM, respectively. The binding was determined from densitometric scans of airfuge-pelleted samples run on Tricine–SDS–polyacrylamide gels and then normalized to the binding of S-1 and peptides to actin in the absence of each other (100%). The actual molar ratio of S-1 bound to actin in the absence of peptides was 0.89 ± 0.05, and that of each of the three peptides in the absence of S-1 was between 3.0 and 6.0. On any given gel, the three peptides showed very similar binding to actin (±25%). However, variations in peptide binding were noted even for the same samples re-run on different gels. These were due to unstable peptide trapping and staining in polyacrylamide gels. Consequently, comparison of peptides bound to actin in the absence and presence of S-1 (and F_{ab} in Table II) was done only with samples run on the same gel. Although these experimental difficulties precluded better estimate of peptide binding to actin, the measurements of relative binding changes, as reported above, were reproducible from gel to gel.

Table II: Binding of $F_{ab}(1–7)$ and Peptides to Actin in the Presence of Each Other[a]

peptide (1.2 mM)	% binding $F_{ab}(1–7)$	% binding peptides
IRICRKG-NH_2	105 ± 5	91 ± 10
IRICRKG	100 ± 5	94 ± 10
RICIRGK	108 ± 6	100 ± 5

[a] The binding of $F_{ab}(1–7)$ and peptides to actin was measured as described in Table I. F_{ab} concentration was 5.0 μM. Its actual binding to actin in the absence of peptides was 0.50 F_{ab}/actin. The binding of F_{ab} to actin in the presence of peptides is normalized to that value. The binding of peptides to actin in the absence of $F_{ab}(1–7)$ was as described in Table I.

effect may be questioned since the scrambled peptide caused similar inhibitions of S-1 binding to actin. More importantly, the actual binding data (legend to Table I) lead to an estimate of between three and six molecules of each peptide bound per actin. Despite a stoichiometry which is clearly greater than one SH_1 peptide per actin subunit, the relative effects of S-1 and peptides on each others binding to actin could be measured with satisfactory accuracy (Table I).

To further test for effects of SH_1 peptides on acto–S-1 interactions, we examined the rate of S-1 modification by NEM in the presence of both SH_1 peptides and F-actin. For this experiment, a Thr-substituted peptide was used to avoid reaction of NEM with the Cys residue in the peptide. This Thr-substituted peptide, like the native sequence, inhibited the maximum Ca-activated force of skinned fibers (Chase et al., 1991). In the presence of both 3.0 μM F-actin and 2.5 mM heptapeptide IRITRKG, both K^+-EDTA- and Ca^{2+}-ATPase activities of S-1 (3.0 μM) were not altered by incubation with 6.0 μM NEM for up to 20 min (data not shown). Thus, unlike $F_{ab}(1–7)$ and ED modification of actin, the peptide did not reverse F-actin's protection of SH_1 in native S-1 from modification by NEM. The peptide did not alter the modification of S-1 by NEM also in the absence of actin.

Probing Peptide Binding to the N-Terminal Segment of Actin with $F_{ab}(1–7)$. As shown in Table II, the binding of $F_{ab}(1–7)$ to actin was not affected by the three peptides. Likewise, the antibody inhibited marginally, if at all, the binding of peptides to actin. Although these results do not

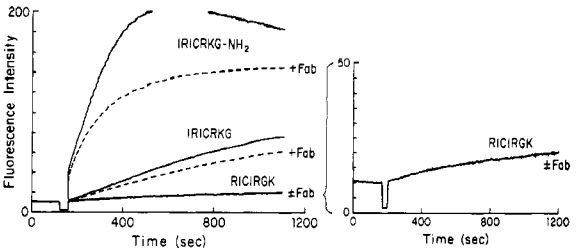

FIGURE 6: Pyrene fluorescence measurements monitoring the polymerization of G-actin by the peptides. G-Actin (4.0 μM) was polymerized by the three peptides (0.2 mM) in the actin depolymerizing solution (0.2 mM $CaCl_2$, 0.5 mM ATP, 0.5 mM β-mercaptoethanol, and 2 mM Tris, pH 7.6) in the absence (solid lines) and presence (dashed lines) of $F_{ab}(1–7)$ (8.0 μM). Inset: polymerization of actin by the scrambled peptide (RICIRGK) shown on an expanded scale.

rule out weak binding of peptides to the N-terminal segment of actin, the existence of such weak binding would not explain the strong inhibition of acto–S-1 ATPase activity by the peptides (Figure 4). The absence of any significant competition between $F_{ab}(1–7)$ and SH_1 peptides suggests that peptide effects on actomyosin are mediated through sites on actin other than the N-terminus. One test of this prediction is based on the observation that SH_1 peptides induce actin polymerization, as does S-1 (Eto et al., 1991). Polymerization of actin by S-1 depends on the initial binding of S-1 to the N-terminal segment of G-actin and is inhibited by $F_{ab}(1–7)$ (DasGupta et al., 1990) or by tryptic cleavage of the 50/20-kDa junction on S-1 (Chen & Reisler, 1991), which binds to the N-terminus of actin (Yamamoto, 1989).

Polymerization of pyrene-labeled actin by the three peptides, monitored by fluorescence, is shown in Figure 6. The ratios of the initial rates of peptide-induced polymerization were 50:5:1 (IRICRKG-NH_2 to IRICRKG to RICIRGK). The differences between the rates of polymerization suggest that both the net charge and the peptide sequence are important for the observed effects. The polymerization of actin by these peptides, in contrast to that by S-1 (DasGupta et al., 1990), did not depend on their binding to the N-terminus of actin. $F_{ab}(1–7)$, which greatly inhibits S-1-induced actin polymerization, has minimal effect on the initial rates of peptide-induced actin polymerization (i.e., corrected for the final extent of actin polymerization achieved in the presence and absence of F_{ab}). Therefore, the polymerization induced by these peptides is mediated through binding to sites other than the N-terminal region of actin. The somewhat lower levels of pyrene fluorescence intensities achieved in the presence of $F_{ab}(1–7)$ are most likely due to decreased macromolecular assembly or actin bundling that may occur in the absence of $F_{ab}(1–7)$. Such an explanation is consistent with a recent observation that $F_{ab}(1–7)$ prevents the bundling of actin by spermine (Cheung, Audit, and Reisler, unpublished results).

DISCUSSION

Our results lead us to conclude the following: (i) actin's N-terminus plays a central role in communication between actin and the SH_1–SH_2 region of native S-1; (ii) cationic peptides from the SH_1–SH_2 region of S-1 bind to actin at more than one site, and functionally significant peptide binding (i.e., ATPase inhibition) is not localized at the acidic N-terminus of actin; (iii) although, as indicated by (ii), the SH_1 peptides do not provide direct information on the function of the SH_1–SH_2 region in S-1, the lack of their binding to the N-terminus of actin implicates this region in indirect rather than direct interaction with the SH_1 site on S-1; (iv) actin

binding peptides which are site-specific and have high affinity for actin will be required for probing actomyosin interactions, particularly for dissecting the molecular domains involved in chemomechanical energy transduction in muscle.

Charge-Dependent Effects of the SH_1 Peptide on Actin. Peptides with sequences derived from the SH_1–SH_2 region of myosin bind to both F-actin (Figure 5 and Tables I and II) and G-actin (Figure 6) and inhibit actin activation of S-1 MgATPase activity (Figure 4), which agrees both qualitatively and quantitatively with previous observations (Suzuki et al., 1987, 1990; Keane et al., 1990; Eto et al., 1990, 1991). Combined with the recent results of Chase et al. (1991), these data demonstrate directly what was previously inferred: peptides with the same sequence and purified in the same manner have an apparently higher inhibitory efficacy in solution assays (acto–S-1 MgATPase activity) compared with skinned fiber assays (maximum Ca-activated force and unloaded shortening velocity). The reason for this difference is not known, but could be related to mechanical constraints imposed upon actomyosin interactions in the myofilament lattice of fibers and in myofibrils which are not present with purified acto–S-1 in solution. Alternatively, the difference in apparent affinities could be related to the regulatory proteins, troponin and tropomyosin, which were present on thin filaments of skinned fibers and myofibrils but which were absent in experiments performed thus far with purified proteins.

Despite quantitative differences in peptide efficacy between solution and fiber assays, inhibition of actomyosin interactions by SH_1 peptides was qualitatively similar in the two assay systems and was dependent on the peptide's net charge more than on the specific sequence (Figure 4; Chase et al., 1991). This conclusion differs from that obtained using rigor binding assays (Suzuki et al., 1987; Eto et al., 1990). However, electrostatic interactions contribute substantially less to the total binding energy of F-actin and S-1 under rigor conditions compared with active crossbridge cycling (Katoh & Morita, 1984; Highsmith & Murphy, 1992). Thus, charge-dependent modulation of acto–S-1 binding by SH_1 peptides would not be as readily discriminated in the rigor binding assay (Table I; Suzuki et al., 1987; Eto et al., 1990). Our conclusion also contrasts with that derived from studies of peptide effects on acto–S-1 ATPase activity where charge- and sequence-specific controls were not carried out (Suzuki et al., 1990; Keane et al., 1990). Evidence that charge is the predominant factor which determines the effects of SH_1 peptides on actomyosin ATPase in solution as in fibers supports the general contention that SH_1 peptides do not necessarily identify the function of the SH_1 sequence in native myosin (Chase et al., 1991).

SH_1 peptide binding to G-actin was also dependent on the net peptide charge (Figure 6). The conclusion that charge is an important parameter is at odds with Eto et al. (1991), but is in accord with previous reports that polymerization of G-actin is also induced by other polyvalent cations such as polylysines (Brown & Spudich, 1979) and the polyamines spermine and spermidine (Grant et al., 1983). In contrast to acto–S-1 ATPase activity, G-actin polymerization also showed an apparent dependence on the peptide sequence (Figure 6). However, we do not interpret the apparent sequence specificity as evidence for a role for the SH_1–SH_2 region in actin polymerization by native S-1 for the following two reasons. First, polymerization by SH_1 peptides was not slowed in the presence of $F_{ab}(1–7)$, a result which contrasts sharply with the antibody's effect on S-1-induced polymerization (DasGupta et al., 1990). Second, kinetic differences between polymerization of G-actin by the isoforms S-1(A1) and S-1(A2) have been attributed to differences in the N-terminus of their respective alkali light chains (Chaussepied & Kasprzak, 1989; Chen & Reisler, 1991; Valentin-Ranc et al., 1991); both isoforms have identical heavy chains, and thus are identical in the SH_1–SH_2 region. Therefore, while it is not surprising that cationic SH_1 peptides induce polymerization of G-actin, there is no evidence for a significant role of the SH_1 region of native S-1 in the S-1-induced polymerization of actin.

The Functional Binding Site or Sites for SH_1 Peptides Is (Are) Not at Actin's N-Terminus. Although SH_1 peptides do not yield information about the function of the SH_1 region in the myosin head, they may provide information about complementary regions on actin. Actin's N-terminus has been implicated as an important component of the actomyosin interface (Sutoh, 1982; DasGupta & Reisler, 1989, 1992; Sutoh et al., 1991), and, with four acidic residues, it is an obvious candidate for binding of cationic peptides. Yet the N-terminus of actin was not the primary functional binding site for SH_1 peptides since binding of both peptides and F_{ab}-(1–7) to F-actin (Figure 5, Table II) or to G-actin (Figure 6) was not mutually exclusive. Additionally, a Thr-substituted peptide did not influence actin's protection of SH_1 in native S-1, although it is clear from our results that this protective effect of actin depends strongly on its N-terminal region. These data, along with the observations that SH_1 peptides are effective inhibitors of acto–S-1 ATPase activity as well as force generation and sarcomere shortening of skinned fibers, imply that other functionally important binding sites for S-1 on actin—in addition to the N-terminus—remain to be identified. The electrostatic nature of peptide binding indicates that, like the N-terminus, this region (or regions) contains acidic residues. However, the observation that more than one peptide binds per actin subunit substantially reduces the utility of SH_1 peptides as specific probes for identifying those domains. A significant challenge is thus to develop new peptide probes which bind to identified regions of actin with higher affinity and greater specificity; such peptides would be useful tools for probing myosin binding regions of actin.

Communication between the N-Terminus of Actin and the SH_1–SH_2 Region of S-1. The mechanism by which actin alters the reactivity of SH_1 has been suggested to be due to steric interaction in which actin covers SH_1, rendering it less accessible to the solvent (Katoh & Morita, 1984). Current estimates of the distance between SH_1 and the actin binding site of S-1 do not provide a conclusive answer on whether SH_1 is close enough to any portion of actin to be consistent with the steric blocking hypothesis. Early cross-linking studies did not reveal any regions of actin in the proximity of the basic residues around SH_1 (Sutoh, 1982). Fluorescence energy-transfer and electron microscopic measurements suggest that the distance between SH_1 and the actin binding site is 4–6 nm (Tokunaga et al., 1987; Botts et al., 1989), in accord with allosteric communication. However, NMR measurements involving S-1 labeled at SH_1 have been interpreted in favor of the steric hypothesis (Barden et al., 1989; Keane et al., 1990). Also, recent cross-linking between MBS-actin and SH_1 on S-1 suggested that the thiol is <1 nm from some portion of the MBS-actin molecule (Bettache et al., 1992). Our work would suggest, but does not prove, that the SH_1–SH_2 region on S-1 and the N-terminal segment of actin interact indirectly in the acto–S-1 complex since there was little or no significant peptide binding to actin's N-terminus. Obviously, more detailed evidence is needed on the molecular structure of the actomyosin interface to distinguish conclusively between

steric and allosteric mechanisms for communication between actin's N-terminus and the SH_1–SH_2 region of S-1.

Nevertheless, it is interesting that introducing positive charges into actin's N-terminus is sufficient to reverse the protective effect of F-actin on the SH_1 site. This result demonstrates that the N-terminus of actin, particularly its charge environment, has a significant role in actin's modulation of SH_1 reactivity on S-1. That environment appears to have little if any impact on the reactivity of the SH_2 which is affected only by the binding of $F_{ab}(1–7)$ to actin. Thus, as with the peptide experiments, these results are consistent with a functionally important S-1 binding role for other regions of actin in addition to its N-terminus (Labbe et al., 1990; Moir et al., 1987). These additional sites have yet to be fully explored in solution and muscle fiber studies.

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