Molecular charge dominates the inhibition of actomyosin in skinned muscle fibers by SH_1 peptides

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ABSTRACT It is not definitively known whether the highly conserved region of myosin heavy chain around SH_1 (Cys 707) is part of the actin-binding site. We tested this possibility by assaying for competitive inhibition of maximum Ca-activated force production of skinned muscle fibers by synthetic peptides which had sequences derived from the SH_1 region of myosin. Force was inhibited by a heptapeptide (IRICRKG) with an apparent $K_{0.5}$ of about 4 mM. Unloaded shortening velocity of fibers, determined by the slack test, and maximum Ca-activated myofibrillar MgATPase activity were also inhibited by this peptide, but both required higher concentrations. We found that other cationic peptides also inhibited force in a manner that depended on the charge of the peptide; increasing the net positive charge of the peptide increased its efficacy. The inhibition was not significantly affected by altering solution ionic strength (100–200 mM). Disulfide bond formation was not involved in the inhibitory mechanism because a peptide with Thr substituted for Cys was inhibitory in the presence or absence of DTT. Our data demonstrate that the net charge was the predominant molecular characteristic correlated with the ability of peptides from this region of myosin heavy chain to inhibit force production. Thus, the hypothesis that the SH_1 region of myosin is an essential part of the force-producing interaction with actin during the cross-bridge cycle (Eto, M., R. Suzuki, F. Morita, H. Kuwayama, N. Nishi, and S. Tokura., 1990, *J. Biochem.* 108:499–504; Keane et al., 1990, *Nature (Lond.)*. 344:265–268) is not supported.

INTRODUCTION

To determine the molecular mechanism of chemomechanical energy transduction by actomyosin, it will be necessary to know the specific amino acids in the myosin primary sequence which contact actin and how the interactions at the actomyosin interface change during the cross-bridge cycle. Three regions of MHC[1] primary sequence have been tentatively identified as actin-binding sites (reviewed by Mornet et al., 1989; Botts et al., 1989; Audemard et al., 1988). Biochemical studies, using a variety of methods, have localized one site at the junction of the 50-kD and 20-kD tryptic fragments and the immediately adjacent COOH-terminus of the 20-kD fragment (Eldin et al., 1990; Kasprzak et al., 1989; Bertrand et al., 1988; Chaussepied and Morales, 1988; Sutoh, 1982, 1983; Mornet et al., 1981*a*, *b*). But this is not the entire actin-binding site (Chaussepied and Kasprzak, 1989; Katoh and Morita, 1984). Chemical cross-linking studies also implicate a second actin-binding region close to the COOH-terminus of the 50-kD tryptic fragment (Sutoh, 1983; Mornet et al., 1981*a*, *b*). The existence of a third site which includes the SH_1 thiol (Cys 707) was proposed by Morita and co-workers from studies of chemical modifications of the S1 fragment of myosin (Suzuki and Morita, 1987; Katoh et al., 1985; Katoh et al., 1984; Katoh and Morita, 1984). Further evidence supporting an actin-binding role of the SH_1 region was obtained from nuclear magnetic resonance (NMR) spectroscopy studies using S1 labeled at SH_1 (Keane et al., 1990; Barden et al., 1989). Most recently, synthetic peptides corresponding to the SH_1 portion of the MHC primary sequence have been found to bind to actin and competitively inhibit both formation of acto-S1 rigor complex (Eto et al., 1990; Suzuki et al., 1987) and actin-activated S1-ATPase activity (Keane et al., 1990; Suzuki et al., 1990). However, there appears to be a significant distance (5 nm) between SH_1 and the actin-binding site measured by electron microscopy (Tokunaga et al., 1987) and by fluorescence resonance energy transfer (FRET) (Takashi and Kasprzak, 1987; Dos Remedios and Cooke, 1984; Trayer and Trayer, 1983; Takashi, 1979). In addition, there is no evidence of chemical cross-linking of the SH_1 region to actin (Bettache et al., 1989; Sutoh, 1982, 1983).

It is important to establish whether the SH_1 region of MHC is part of the actin-binding site for interpretation of existing data, such as the functional significance of

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[1]*Abbreviations used in this paper:* ATP, adenosine 5′-triphosphate; CK, creatine kinase; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; MHC, myosin heavy chain; MOPS, 3-[N-morpholino]propanesulfonic acid; pCa = $-\log_{10}[Ca^{2+}]$; PCr, phosphocreatine; Pi, orthophosphate; RP-HPLC, reverse phase high performance liquid chromatography; S1, myosin subfragment-1; SH_1, Cys 707 of MHC; SH_2, Cys 697 of MHC; μ, ionic strength; V_{us}, velocity of unloaded shortening.

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motion of reporter groups covalently attached to SH_1 (reviewed by Thomas, 1987), and to focus further structure-function studies at the molecular level. We approached the problem of whether SH_1 is at the actin interface by testing, on skinned muscle fibers, for inhibitory properties of synthetic peptides that correspond to the SH_1 sequence of MHC. This is a logical extension of this search for functional domains in the primary sequence of MHC, as described above (Eldin et al., 1990; Keane et al., 1990; Suzuki et al., 1987; Katoh et al., 1985). Skinned fibers make a useful assay system for determining the physiological significance of putative sites of interaction between actin and myosin, and they have been studied with this in mind by Keane et al. (1990). Small peptides (the peptides described herein were on the order of 1 kD) can be used to probe the actomyosin interaction in skinned fiber experiments, because there are no membrane barriers to impede diffusion throughout the myofilament lattice. As in living muscle, the actomyosin interaction in skinned fibers is structurally constrained by the myofilament lattice and the exernal mechanical connections. Concomitant with ATP hydrolysis, the actomyosin interaction is the basis for, and is in turn influenced by, isometric force and sliding of the thick and thin filaments, physiologically relevant parameters that are not present or controlled in most in vitro experiments. If actin-binding peptides associate with, and thus block a part of actin which is important for cross-bridge function, then force generation and movement by actomyosin should either be partially or wholly inhibited. Inhibition should be evident whether the region probed is required for one step in a sequence that leads to force generation (Huxley and Simmons, 1971) or whether it is one of many regions which together are responsible for generation of force. This allowed a test of whether the SH_1 sequence in the myosin head, which corresponds to actin-binding peptides, is an essential part of the actin-binding site in the native molecule.

Portions of this work have appeared elsewhere in preliminary form (Chase et al., 1991; Chase and Kushmerick, 1991; Chase et al., 1990).

MATERIALS AND METHODS

Skinned fibers

Single cell segments were dissected from bundles of glycerinated fibers from rabbit psoas muscle (Chase and Kushmerick, 1988a). The ends of the fiber segments were wrapped in aluminum foil "T-clips" (Goldman and Simmons, 1984). Before insertion in the clips, the segment ends were chemically fixed by microapplication of glutaraldehyde (5% in H_2O, with 1 mg/ml Na-fluorescein); the dissection bath had 10 mg/ml of soybean trypsin inhibitor added to restrict diffusion of the fixative. The fiber segment was mounted in a bath at the focal plane of an inverted microscope (Leitz, Wetzlar, Germany), with the clips attached to hooks on a motor (General Scanning, Watertown, MA) at one end and a force transducer (Cambridge Technology, Watertown, MA) at the other end. Sarcomere length was monitored visually, both in relaxing and activating solutions, and was also monitored by laser diffraction in some experiments. The solution holders (100 or 300 μl) were made of electroplated aluminum and had glass bottoms; the temperature of the bath was maintained at 12°C using thermoelectric chips (Cambion, Cambridge, MA). Solution changes were effected by raising the fiber out of one bath allowing another bath to be rotated under it, and lowering the fiber into the new bath.

During the experiment, the fiber segment was slackened every 5 s by shortening the fiber at a rate approximately equal to the maximum shortening velocity, and then rapidly restretching it to the original length (Brenner, 1983). Force measurements or length steps (as in determination of V_{us}) were performed during the steady-state period between releases (Sweeney et al., 1987). Signals were low-pass filtered (f_c = 4 kHz) and records were digitized at rates of up to 12.5 kHz per channel. Force and V_{us} measurements in the presence of peptide were normalized to the average of bracketing control measurements made before and after.

Solutions

The bathing solutions were made and the compositions calculated as previously described (Chase and Kushmerick, 1988*a*). The standard conditions were (mM) 5 MgATP, 15 PCr, 1 Pi, 4 EGTA, 50 DTT, 250 MOPS, μ = 200 adjusted with acetate and Tris, 50 Na^+, 50 K^+, 3 Mg^{2+}, pCa 4.0–4.5 (activating) or pCa > 8 (relaxing), pH 7.1, 250 U/ml CK, 12°C. In experiments where μ was varied, the following changes were made in all solutions, in addition to the variation in acetate and Tris: 4 MgATP, 10 PCr, 40 MOPS, 36.5 Na^+, and 33.5 K^+. No account was taken of the peptides' contribution to μ. The solutions were made up as concentrated stocks (1.5 times) and were diluted to final volume by adding appropriate amounts of concentrated peptide solution (50 mg/ml) and H_2O.

Peptides

Peptides were synthesized by Applied Biosystems (Foster City, CA) or by the Howard Hughes Chemical Synthesis Facility, University of Washington (Seattle, WA). By convention, all peptide sequences are given from NH_2- to COOH-terminus; acetylation of the NH_2-terminus is indicated by Ac- and amidation of the COOH-terminus is indicated by $-NH_2$. For purification, a C_{18} 300 Å semipreparative RP-HPLC column was used (model 201TP510, Vydac, Hespera, CA). The peptides were dissolved in a two- to three-fold molar excess of both DTT and MOPS (pH 7.0). Separation was achieved by an elution gradient from 5–60% acetonitrile (ACN) in H_2O with 0.1% trifluoroacetic acid (TFA). The fractions containing the correct peptide sequence were collected in acid-washed glassware and lyophilized. The lyophilized peptides were redissolved in 5% acetic acid and identical fractions from several HPLC separations were combined and lyophilized. Lyophilization was repeated once or twice more, dissolving the peptides in H_2O until all fractions containing the same peptide had been combined into one.

We verified that the peptides were not altered during the purification procedure by the following criteria: (*a*) molecular mass and amino acid composition for each peptide were the same as theoretical; (*b*) retention time and purity of each peptide were checked by analytical RP-HPLC (201TP54 Vydac or 11793 Waters Chromatography Div., Milford, MA) with detection by absorbance at 214 nm. Molecular mass was determined by fast atom bombardment mass spectroscopy (FAB/MS) using a VG 70 SEQ tandem hybrid instrument (VG Analytical,

Altringham, UK). Amino acid composition was determined by the Waters Pico-Tag method after 24 h vapor phase acid hydrolysis (Bidlingmeyer et al., 1984). In some cases, electron probe x-ray microanalysis (EPXMA; Johnson and Cantino, 1986) and ^{19}F-NMR were performed to assay for contaminants. We found that RP-HPLC purification of the starting material not only removed contaminating peptide sequences and other synthesis byproducts but also removed inorganic material, including silicon, fluoride, and aluminum, from peptides synthesized by the *t*-Boc strategy. Aluminofluoride at concentrations comparable to that found in some unpurified peptides markedly inhibited force production (Chase and Kushmerick, 1990).

Myofibrils

Myofibrils were made from glycerinated psoas muscle from rabbit (Knight and Trinick, 1982). Muscle strips were tied to wooden sticks at in vivo length and immersed in chilled myofibril glycerinating buffer (13.3 mM KH_2PO_4, pH 7.0 at 0°C, mixed 1:1 with glycerol); after 1 h, the muscle strips were transferred to fresh myofibril glycerinating buffer and stored at −20°C. To make myofibrils, a muscle strip was minced in 10 vol of ice cold myofibril rigor buffer (in mM; 100 KCl, 2 $Mg(CH_3CO_2)_2$, 1 EDTA, 10 H_3PO_4, 0.5 DTT, pH 7.0 at 0°C) and then homogenized. The homogenate was centrifuged at 2,500 *g*, the supernatant discarded, and the pellet resuspended in myofibril rigor buffer; centrifugation and resuspension were repeated three times. Myofibrils were mixed with an equal volume of chilled glycerol and stored at −20°C for up to 8 mo. Protein concentration was determined from the absorbance at 280 nm of an aliquot of myofibrils dissolved in 1% Na-lauryl sulfate (SDS) (corrected for scattering by subtraction of the absorbance at 310 nm), using an extinction coefficient of 0.7 $ml \cdot mg^{-1} \cdot cm^{-1}$.

Myofibrillar ATPase activity was determined by the pH-stat method (White, 1982) using an autotitrator (Radiometer America, Cleveland, OH). Solutions and conditions for myofibrillar ATPase determinations were essentially identical to those used for fiber experiments, except that the pH buffer concentration was reduced to 5 mM. The titrant (in mM; 5 KOH and 195 KCl) was degassed and kept under CO_2 adsorbant. ATPase rate per myosin S1 was calculated by assuming 0.82 μmol myosin/g of myofibrillar protein (Yates and Greaser, 1983) and a stoichiometry of 0.58 mol H^+ released per mol ATP hydrolyzed (pH 7.1).

RESULTS

Maximum Ca-activated force of skinned fibers from rabbit psoas muscle was reversibly inhibited in the presence of a synthetic peptide with the sequence IRICRKG with an apparent $K_{0.5}$ of ~4 mM (Fig. 1). V_{us} of skinned fibers and MgATPase activity of fully Ca-activated myofibrils were also inhibited by purified peptide IRICRKG, although the apparent $K_{0.5}$'s were both higher than for inhibition of force (Fig. 2). The shift between the isometric force and V_{us} curves (Figs. 1 and 2) is a real difference because both measurements were made on each fiber during the same activation by calcium. Isometric force was obtained from the force level at the beginning of the digitized slack test records, before the slack release of the fiber, which allowed an unambiguous determination of the force baseline.

Four RP-HPLC purified peptides, variants on the sequence IRICRKG, were used to test the extent to which the peptide's amino acid sequence specified its inhibitory properties. The first was the very highly conserved 16 amino acid sequence around and including

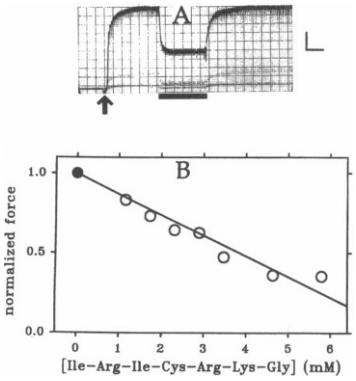

FIGURE 1 Synthetic peptides derived from the MHC sequence around SH_1 reversibly inhibited maximum Ca-activated force of skinned muscle fibers. (*A*) Original force record demonstrating inhibition of force in the presence of peptide. Calibrations: force, 20 mg; time, 1 min. At the arrow, the solution was changed from relaxing (pCa 8) to activating (pCa 4.5). Transients in the force record result from periodic length releases followed by restretch (see Methods). The bar beneath the record indicates the period during which 3.4 mM peptide IRIPRKG was present in activating solution. The inhibitory properties of other peptides were qualitatively similar to that shown. (*B*) Steady-state isometric force of single skinned fibers from rabbit psoas muscle in the presence of IRICRKG was normalized to the average of bracketing contractions in the absence of peptide (●), as shown in *A*. Each point represents a single determination. The line was drawn according to the least squares regression on the data for the equation $y = 1 - k \cdot x$(k = 0.130; 0.007 SE).

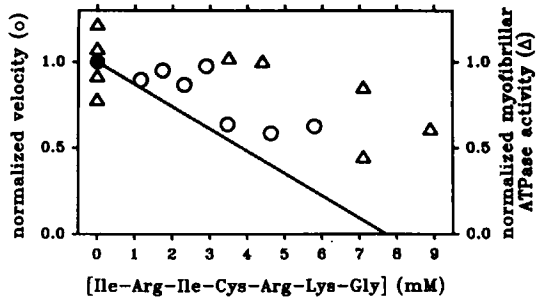

FIGURE 2 Unloaded shortening velocity of single skinned fibers (○) and maximum Ca-activated ATPase activity of myofibrils (△) were inhibited by synthetic peptide IRICRKG. V_{us} (○), measured during the same activations as isometric force determinations (Fig. 1), was normalized to the average of bracketing contractions in the absence of peptide (●). Myofibrillar ATPase activity (△), measured by the pH-stat method, was normalized to the average of four determinations made in the absence of peptide for comparison with fiber force and V_{us} measurements. Each point represents a single determination. The line was redrawn from Fig. 1 *B*.

SH_2 and SH_1 (Ac-CNGVLEG**IRICRKG**FP-NH_2) (Elzinga and Collins, 1977; Warrick and Spudich, 1987). It was expected that the longer peptide might have greater affinity for actin and that chemically blocking the charged ends might further enhance any correspondence to the native MHC structure induced by binding of the peptide to actin. But the apparent $K_{0.5}$ derived from force measurements on skinned fibers was similar to that for the heptapeptide (Fig. 3). Proline, substituted for cysteine (IRIPRKG), was expected to disrupt any induced structure in the peptide. Again, there was no substantial change in the apparent $K_{0.5}$ obtained from the skinned fiber assay (Fig. 3). The third peptide we used had the same seven amino acids as the native heptapeptide, but in a scrambled order (RICIRGK). If the native sequence peptide truly mimicked an actin-binding function of this highly conserved region of MHC, then reordering the residues should render it less effective. Again, the apparent $K_{0.5}$ of force inhibition was ~4 mM (Fig. 3). We obtained a small but significant difference between IRICRKG (Fig. 1) and the "blocked" heptapeptide, Ac-IRICRKG-NH_2 ($K_{0.5}$ ~ 6 mM; Fig. 4), which was used as the control sequence for the experiments shown in Figs. 4–6. However, for comparison between peptides, it is important to note that the data presented in Figs. 1–3 were obtained during one series of experiments, whereas the data shown in Figs. 4–6 were collected during a second series. We concluded that the inhibition of force by these peptides does not depend on a specific coding of information in the amino acid sequence. The common feature of these five peptides is that they all have the same net charge (3^+).

To determine whether the net charge of the peptides influenced their inhibitory properties, two other peptides were tested: IRICRKG-NH_2 (net charge 4^+) was a more effective inhibitor of force than the native sequence peptide, while peptide IEICRKG (net charge 1^+) was less effective (Fig. 4). Chemically blocking only the COOH-terminus (IRICRKG-NH_2), thus increasing the net charge on the peptide by 1^+, resulted in greater inhibition at lower concentrations when compared with either Ac-IRICRKG-NH_2 (Fig. 4) or IRICRKG (Fig. 1). The significance of the difference between peptides of various charges is strengthened by the fact that two or

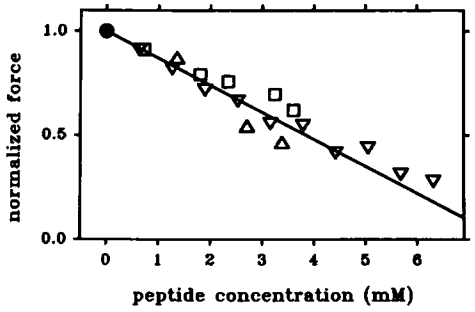

FIGURE 3 Inhibition of maximum Ca-activated force by synthetic peptides Ac-CNGVLEGIRICRKGFP-NH_2 (□), IRIPRKG (△), and RICIRGK (▽). Steady state isometric force of single skinned fibers from rabbit psoas muscle was normalized to the average of bracketing contractions in the absence of peptide (●). Each point represents a single determination. The line was redrawn from Fig. 1 *B*.

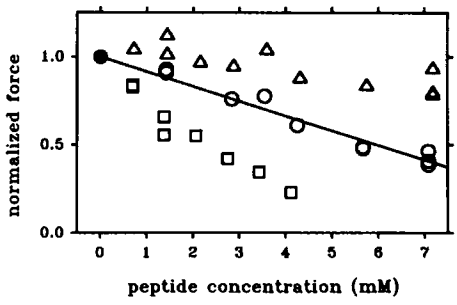

FIGURE 4 Inhibition of maximum Ca-activated force by synthetic peptides IRICRKG-NH_2 (□), Ac-IRICRKG-NH_2 (○), and IEICRKG (△). Steady-state isometric force of single skinned fibers from rabbit psoas muscle was normalized to the average of bracketing contractions in the absence of peptide (●). Each point represents a single determination. The line was drawn according to the least squares regression on the data from peptide Ac-IRICRKG-NH_2 (○) for the equation $y = 1 - k \cdot x$(k = 0.084; 0.003 SE).

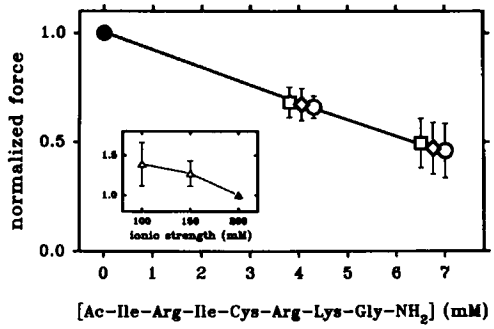

FIGURE 5 Inhibition of maximum Ca-activated force by synthetic peptide Ac-IRICRKG-NH_2 at ionic strengths 100 mM (□), 150 mM (◇), and 200 mM (○). Steady-state isometric force of single skinned muscle fibers was normalized to the average of bracketing contractions in the absence of peptide and at the same μ (●). As shown in the inset, force (in the absence of peptide) increased as μ was decreased; in the inset, force was normalized to the average of bracketing contractions at μ = 200 mM (▲). Ionic strength was varied by changing the concentration of Tris cation and CH_3COO^- anion. Points are the mean ± SD (N = 3 in the main figure; N = 12 in the inset). For clarity, the abscissa was shifted by −0.25 mM for the data at μ = 100 mM, and by 0.25 mM for the data at μ = 200 mM. All lines were drawn to connect the points.

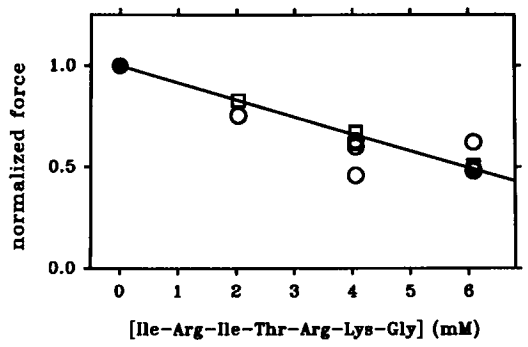

FIGURE 6 Inhibition of maximum Ca-activated force by synthetic peptide IRITRKG in the presence (○) and absence (□) of DTT (50 mM). Steady-state isometric force of single skinned muscle fibers was normalized to the average of bracketing contractions in the absence of peptide and at the same concentration of DTT (●). Each point represents a single determination. The line was redrawn from Fig. 4.

three different peptides were tested on each fiber shown in Fig. 4. Thus, cationic SH_1 peptides inhibited maximum Ca-activated force in a manner that depended on the charge of the peptide; more positively charged peptides were more effective inhibitors.

Lowering ionic strength from 200 mM to 150 or 100 mM by reducing the concentrations of $Tris^+$ and CH_3COO^- did not alter the inhibitory effects of the native sequence peptide (Fig. 5). Note that in Fig. 5, all force values have been scaled to the control force at the same μ; the decrease of control force with increased μ obtained in this set of experiments is shown in the inset. While there was no difference in the normalized data at the three μ's, the observed change in absolute force obtained at a given peptide concentration was greater at the lower μ's due to the higher absolute force. It is clear that the ionic strength contribution of the peptide (and its associated anion) is not sufficient to explain its inhibitory activity. For example, 4 mM of a trivalent ion (with three monovalent counterions) would increase μ by 24 mM. This would correspond to, at most, an 11% decrease in force (Fig. 5, *inset*), whereas the actual decrease was 33 to 50%. In addition, the actual contribution to μ of a peptide with net charge 3^+ is probably less than that of a trivalent ion.

We examined the effects of a peptide in which threonine was substituted for cysteine as a control for disulfide bond formation, either between peptides (dipeptide formation) or between peptides and the native contractile proteins, which might have occurred despite the use of a reducing agent. This variation in sequence occurs naturally in an invertebrate myosin (Warrick et al., 1986). The Cys→Thr substituted peptide also inhibited force, as did the native sequence peptide (Fig. 6). The inhibition was not significantly different in the presence or absence of DTT (Fig. 6), demonstrating that inclusion of DTT in the bathing solutions did not unexpectedly modify the previous results.

DISCUSSION

Peptides with sequences derived from the MHC primary sequence around SH_1 reversibly inhibited maximum Ca-activated force production and unloaded shortening velocity of skinned fibers from rabbit psoas muscle, and also inhibited maximum Ca-activated myofibrillar ATPase activity. Although these results coincide with predictions for a site-specific inhibitor of the actomyosin interaction (Keane et al., 1990; Suzuki et al., 1987), the fact that force inhibition depended on the peptide's net charge rather than on the specific sequence of amino acids leads us to conclude the opposite. Our data do not support the hypothesis that these peptides from the region of SH_1 are structurally specific inhibitors of the force-generating interaction between myosin and actin, and thus do not support the inference that the SH_1 region is an essential part of the interface with actin. This interpretation contrasts markedly with the conclusion of recent studies involving SH_1 peptides that the function (or functions) of the SH_1 region of MHC entails binding to actin (Suzuki et al., 1987; Suzuki et al., 1990; Keane et al., 1990; Eto et al., 1990).

Previous work suggested that SH_1 is at or near the interface with actin, although the functional significance of the proposed interaction differs. Morita and co-workers postulated that SH_1 is part of the strong-binding site (the "S-site", which corresponds to residues 702–708 of the numbering scheme used by Keane et al., 1990; Suzuki et al., 1987; Suzuki et al., 1990; Eto et al., 1990). According to Trayer and co-workers (Keane et al., 1990), the residues immediately adjacent to the SH_1 Cys (residues 687–717 according to the same numbering scheme) were proposed to be directly involved in modulation of thin filament Ca-sensitivity (Keane et al., 1990). Peptide Y669 (comprised of residues 706–715 according to the same numbering scheme) was particularly effective at enhancing Ca-sensitivity of both regulated acto-S1 ATPase and skinned fiber force. A second overlapping region, comprising the SH_1 Cys and additional COOH-terminal residues, was also proposed to be part of the actomyosin interaction which is central to acto-S1 ATPase activity (Keane et al., 1990). Although Keane et al., also noted some inhibition of skinned fiber force development by the same peptides, the results were not presented in detail. Our primary experimental goal was to probe the force-generating interface between myosin and actin, and thus, the majority of experiments were carried out using fully Ca-activated skinned fibers.

Maximum Ca-activated force was chosen as the primary indicator because peptide binding to a myosin contact site on actin should reduce the force-generating potential with the magnitude of the effect being proportional to the number of force-generating cross-bridges, which is greatest at full Ca-activation. The control peptides used for the conclusions of Keane et al. (1990) and the initial conclusions of Suzuki et al. (1987, 1990) were based on sequences derived from adjacent regions of MHC. Our data suggest that this may not be sufficient to discriminate functional regions of primary sequence, and that SH_1 is not part of the force-generating interface between myosin and actin. Therefore, we also suggest that any role for SH_1 in modulating the cooperativity of ATPase and force development at submaximal $[Ca^{2+}]$ should be regarded as speculative until appropriate controls for sequence specificity are made.

Peptides appear to be less effective inhibitors in fiber and myofibril assays than in assays conducted with purified proteins. The apparent inhibitory constant obtained in this work (4–6 mM for skinned fiber force) differed significantly from that obtained in solution by Suzuki et al. (230 μM for rigor binding, Suzuki et al., 1987; 47 μM for actin-activated MgATPase activity, Suzuki et al., 1990), although peptides with the same sequence were used in all of these studies. This difference of 1–2 orders of magnitude was not due to differences in charges at the ends of the peptides (compare Figs. 1 and 4). Additional protein (BSA or CK) was present in both experiments, which would compensate for nonspecific protein binding and counteract the possible influence of any endogenous proteolytic enzymes that may have remained in the preparation (we also included trypsin inhibitor in the dissection bath, although for a different reason: see Methods). Differences in ionic strength do not seem likely to be the complete explanation since the apparent $K_{0.5}$ of the peptide in our assays was not altered when μ was lowered from 200 to 100 mM, which is slightly lower than μ used in the rigor binding assays (Suzuki et al., 1987), although it is not as low as that used in the ATPase assays with purified proteins (Suzuki et al., 1990). Other related peptides were tested by Keane et al. (1990) using both solution assays and fiber assays. Apparent inhibitory $K_{0.5}$'s for the fiber work were not given, but comparing two peptides common in both of their Figs. 3 and 4 (peptides Y629 and Y630) indicates that at least an order of magnitude higher peptide concentration was required for half-maximal inhibition of maximum Ca-activated force in skinned fibers compared with that necessary to inhibit acto-S1 ATPase activity. The fact that analogous results were obtained with various peptide sequences in different laboratories suggests that the quantitative difference between peptide effects on purified proteins vs. in fibers is a valid observation that is not yet explained. A difference in the major anion (acetate in our experiments, whereas Suzuki et al. [1987, 1990] used chloride) does not seem to be the likely causal factor, in spite of destabilization of myosin structure by Cl^- that occurs at much higher $[Cl^-]$ (Stafford, 1985), because Keane et al. (1990) used Cl^- in all of their experiments. The use of purified proteins and protein fragments to examine the actomyosin interaction in solution confines the investigation to that interface which exists under essentially "unloaded" conditions (Suzuki et al., 1987, 1990; Keane et al., 1990; Eto et al., 1990). The "unloaded" nature of the actomyosin interaction in the solution assays cannot explain the difference because peptide inhibition of both V_{us} of fibers and ATPase activity of myofibrils had a higher, rather than lower, apparent $K_{0.5}$ than force inhibition. Furthermore, the charge of the intact myofibrillar structure cannot be responsible for the difference because the negative Donnan potential of fibers and myofibrils would result in an elevated concentration of cationic peptide within the fiber lattice relative to the bath concentration, which should have given an apparent lowering of $K_{0.5}$ in fibers and myofibrils compared with proteins in solution. Overall, these results suggest significant unexplained differences may exist between the mechanically unconstrained interaction between the purified contractile proteins in solution and the actomyosin interaction within the myofilament lattice.

The involvement of charge in the inhibitory mechanism of SH_1 peptides should not be surprising in light of the well-known part played by ionic interactions in cross-bridge function (e.g., Katoh and Morita, 1984; Sutoh, 1982; Gordon et al., 1973). Also, numerous nonpeptide inhibitors of the actomyosin interaction, including salts, have been identified; even uncharged molecules such as 2,3-butanedione monoxime and sucrose inhibit force and velocity of skinned fibers (Fryer et al., 1988; Chase and Kushmerick, 1988*a*), although inhibitory sucrose concentrations were two orders of magnitude higher than the concentrations of peptides used in these experiments. We noted that there was also a correlation between inhibition of actin-activated MgATPase activity of S1 and net peptide charge in the data of Keane et al. (1990). In their paper, those peptides which were most inhibitory were synthesized according to the MHC sequence including and immediately adjacent to the SH_1 cysteine and were purified using gel filtration and ion-exchange chromatography; those which were least inhibitory came from the sequence flanking the SH_1 region and were purified by RP-HPLC. Purification methods could conceivably make a difference because, in our work, we found that inorganic contaminants significantly altered the apparent

$K_{0.5}$ (Chase and Kushmerick, 1989, 1990; Chase and Kushmerick, 1988*b*), but the major correlating property was charge. Within the former (inhibitory) group of peptides studied by Keane et al. (1990), the most inhibitory peptide, GIRICRKGFPSRILYADFKQRYKVLNAS (Y630; the SH_1 Cys is bolded for reference), had a net charge of 6^+, assuming that both the NH_2- and COOH-termini were chemically blocked or both were free. The least inhibitory peptide of this group, GVLEGIRICR (Y668; the SH_1 Cys is bolded for reference), had a net charge of 1^+. Peptides with charges 2^+ to 3^+ had intermediate inhibitory effects. The latter "flanking" peptides, which were the least inhibitory of all, had net charges of 1^- to 1^+. Suzuki et al. (1990) also examined the effects of two peptides on actin-activated MgATPase activity of S1; in their data, the longer SH_1 peptide, with a net charge of 4^+, was inhibitory at lower concentrations than the second, shorter peptide with a net charge of 2^+. Despite the correlation between charge and inhibition of actively cycling cross-bridges, inhibition of rigor complex formation between actin and S1 was demonstrated to depend on amino acid composition more than net peptide charge of peptides with variations in individual amino acids (Eto et al., 1990).

The high sequence homology of the SH_1 region in myosins from a wide variety of organisms (Stedman et al., 1990; Warrick and Spudich, 1987), combined with the extensive literature on the biochemistry of SH_1, justify the supposition that it has an important role in myosin function. Our data do not support an actin-binding role for the SH_1 region as a necessary part of cross-bridge function. Further tests should therefore be focused on alternative possibilities for the function of the SH_1 region of myosin and on which portions of the MHC primary sequence are involved in binding to actin.

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