Role of Sequence 18–29 on Actin in Actomyosin Interactions†

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Received February 1, 1993; Revised Manuscript Received March 15, 1993

ABSTRACT: Affinity-purified polyclonal antibodies prepared against a synthetic peptide corresponding to sequence 18–29 from the N-terminus of rabbit α-skeletal actin reacted with G- and F-actin. Epitope mapping experiments with thrombin and hydroxylamine cleaved actin, and immunochemical assays verified the specificity of antibodies for the 18–29 sequence on actin. The binding of up to 0.5 mol of IgG per mole of actin did not affect the rigor binding of myosin subfragment 1 (S-1) to actin. Similarly, the binding of IgG to actin was not changed by a complete saturation of actin by S-1. In contrast to this, the weak acto–S-1 interactions in the presence of ATP were strongly inhibited by the 18–29 antibodies. At 25 °C, the acto–S-1 ATPase activity was inhibited by IgG stronger than the binding of S-1·ATPγS to actin. Thus, at this temperature, a catalytic inhibition of the acto–S-1 system appears to account at least in part for the antibody effect. Acto–S-1 ATPase activities at 25 °C were inhibited also by F_{ab}(18–29). At 5 °C, the acto–S-1 ATPase activity and the binding of S-1·ATP to actin were inhibited approximately to the same extent by IgG(18–29). These results are discussed in terms of S-1 binding sites on actin and the possible role of sequence 18–29 in actomyosin interactions.

Recent advances in the understanding of monomeric and filamentous actin structures (Kabsch *et al.*, 1990; Holmes *et al.*, 1990; Milligan *et al.*, 1990; Kabsch & Vandekerckhove, 1992) provided a strong impetus for the mapping of myosin binding sites on actin. The goal of such studies is to obtain a detailed description of the actomyosin interface and its evolution during adenosine 5′-triphosphate (ATP)[1] hydrolysis. As a first and major step toward this goal, the actomyosin interface must be defined for the two main states of the complex: the strongly (rigor) and the weakly bound (in the presence of ATP) actin and myosin. Since the transition between these states is considered to be a key step in the contractile cycles [e.g., see Brenner (1990) and Eisenberg and Hill (1985)], the structural information about them is vital to the understanding of energy transduction in actomyosin-based processes.

Structural considerations (Kabsch *et al.*, 1990; Labbe *et al.*, 1990; Kabsch & Vandekerckhove, 1992) have led to the suggestion that myosin heads contact actin at its N-terminal acidic residues 1–4 and along the loop 20–28, the helix 79–93, the hydrophobic residues in the helix (340–350), and the C-terminal helix (360–364). Several immunochemical (Mejean *et al.*, 1987; Miller *et al.*, 1987; DasGupta & Reisler, 1989, 1992), biochemical (Bertrand *et al.*, 1989; Moir *et al.*, 1987; Van Eyk *et al.*, 1991), and mutagenic studies (Sutoh *et al.*, 1991; Cook *et al.*, 1992; Aspenström & Karlsson, 1992) clarified the role of actin's N-terminal residues in actomyosin interactions. It may be concluded from these investigations that the N-terminal acidic residues on actin are critical to its ability to activate the ATPase activity of myosin and, thus, to the contractile function of actin. Yet, the same residues are not essential for rigor or even weak binding of myosin to actin (Reisler, 1993). A convincing verification of this conclusion has been obtained most recently by showing that addition of two extra negatively charged residues to the N-terminus of wild-type yeast actin increases 3-fold the V_{max} of acto–S-1 ATPase but does not change its K_{ATP} value (Cook *et al.*, 1993).

Of the two possible "strong" myosin binding sites on actin, the loop (20–28) and α-helix (340–350) (Kabsch *et al.*, 1990; Kabsch & Vandekerckhove, 1992), the former site has been implicated in rigor actomyosin interactions also by solid-phase immunochemical assays (Mejean *et al.*, 1987) and NMR experiments (Moir *et al.*, 1987). A contact between residues 18–28 and myosin has been suggested also by a peptide mimetic approach, in which several peptides spanning different portions of the 1–28 sequence on actin were examined for interactions with myosin (Van Eyk *et al.*, 1991). However, the possible role of 18–28 residues on actin in weakly bound actomyosin was not examined in these studies, and the importance of the 18–28 sequence to rigor interactions in solution was not evaluated.

In this work, we have employed affinity-purified polyclonal peptide antibodies against the 18–29 sequence on actin as a probe of this region's role in rigor and weak actomyosin interactions. We show that while the 18–29 sequence on actin, or at least part of it, is not necessary for rigor binding of myosin to actin, it is important for actomyosin interactions in the presence of ATP. Preliminary results of this study were reported before (Adams & Reisler, 1992).

MATERIALS AND METHODS

Reagents. Distilled and Millipore-filtered water and analytical-grade reagents were used in all experiments. ELISA plates (Dynatech Immulon I) and hydroxylamine hydrochloride were purchased from Fisher Scientific Co. Papain, thrombin, 4-chloro-1-naphthol, alkaline phosphatase substrate 104, alkaline phosphatase conjugated goat anti-rabbit IgG, peroxidase-conjugated goat anti-rabbit IgG, protein A, cy-

† This work was supported by USPHS Grant AR22031 and NSF Grant MCB9206739.

[1] Abbreviations: S-1, myosin subfragment 1; 18–29 peptide antibodies, affinity-purified IgG and F_{ab} fragments of polyclonal antibodies directed against sequence 18–29 (KAGFAGDDAPRAY) from the N-terminus of rabbit α-skeletal actin; F_{ab}(1–7), F_{ab} fragment of affinity-purified antibodies against the N-terminal residues 1–7 on rabbit α-skeletal actin; ELISA, enzyme-linked immunosorbent assay(s); ATP, adenosine 5′-triphosphate; ATPγS, adenosine 5′-*O*-(3-thiotriphosphate); ATPase, adenosinetriphosphatase.

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anogen bromide activated Sepharose, Freund's adjuvent, TLCK-treated α-chymotrypsin, iodoacetic acid, ATPγS, and ATP were purchase from Sigma Chemical Co. (St. Louis, MO). Keyhole limpet hemocyanin and Aquacide II were purchased from Calbiochem (La Jolla, CA). The synthetic peptide used for immunization of rabbits was purchased from the custom peptide synthesis facility at the University of California at San Diego.

Preparation of Proteins. Rabbit skeletal muscle actin was prepared in G-buffer (0.5 mM β-mercaptoethanol, 0.2 mM ATP, 0.2 mM $CaCl_2$, and 5 mM Tris, pH 7.6) by the procedure of Spudich and Watt (1971). Subfragment 1 (S-1) was prepared by chymotryptic digestion of myosin (Weeds & Pope, 1977). S-1 was dialyzed, just prior to use, into a low-salt buffer (10 mM KCl/10 mM imidazole, pH 7) which favored S-1 binding to actin. Myofibrils were prepared by the method of Kominz (1970). $F_{ab}(1-7)$, the antibody against the N-terminal residues 1–7 on α-skeletal actin, was a generous gift from Dr. DasGupta.

Preparation of Peptide Antibodies. The peptide Lys-Ala-Gly-Phe-Ala-Gly-Asp-Asp-Ala-Pro-Arg-Ala-Tyr, corresponding to residues 18–29 of skeletal muscle α-actin, was used for immunization. This sequence constitutes an invariant region in actin isoforms (Vandekerckhove & Weber, 1984). [^{14}C]-Glycine was incorporated into the peptide in either the Gly-20 or the Gly-23 position. The C-terminal Tyr residue was included to facilitate coupling of the peptide to a carrier protein, keyhole limpet hemocyanin (KLH), as described by Otey *et al.* (1986). Coupling efficiency ranged between 50 and 70% as determined by radioactivity of the ^{14}C-labeled peptide. Female New Zealand white rabbits were immunized with 500 μg of peptide–KLH conjugate mixed in equal parts with Freund's adjuvant as described by Otey *et al.* (1986).

Preparation of Affinity-Purified 18–29 Peptide Antibodies. The IgG component of serum was isolated by adsorption to protein A–Sepharose CL-4B (Reeves *et al.*, 1981; Kilion & Holtgrewe, 1983) and elution with 0.1 M glycine at pH 3.0. IgG specific for the actin peptide was purified by affinity chromatography using G-actin covalently coupled to Sepharose. The resin was prepared as described by Miller *et al.* (1987). Following elution with 0.1 M glycine (pH 3.0), the specific IgG was concentrated with Aquacide II [(carboxymethyl)-cellulose] and then dialzyed against phosphate-buffered saline (PBS) or 10 mM KCl/10 mM imidazole, pH 7.0. Peptide-specific Fab fragments were prepared by papain digestion of specific IgG (Miller *et al.*, 1987). Uncleaved IgG and F_c fragment were removed by adsorption to a protein A–Sepharose CL-4B column. The Fab was concentrated with Aquacide II and dialyzed against either PBS or 10 mM KCL/10 mM imidazole, pH 7.0, containing 2 mM iodoacetic acid.

Immunological Assays. Enzyme-linked immunosorbent assays (ELISA) were performed as described by Atherton and Hynes (1981) and DasGupta *et al.* (1990). Antibody titers were determined by using microtiter plates coated with skeletal muscle G-actin at 5 μg/well. Reactivity of the 18–29 antibody with monomeric and polymerized actin was compared by using microtiter plates coated with either 100 μg/mL G-actin or 100 μg/mL F-actin solutions. Competitive ELISA were performed by adding to microtiter plates coated with either 10 μg/well of G- or F-actin a fixed amount of antibody (noted in the figure legends) in the presence of the 18–29 peptide (0–60 μM). The competing peptide and antibodies were added to triplicate wells in either G-actin buffer (5 mM Tris, 0.2 mM $CaCl_2$, 0.5 mM β-mercaptoethanol, and 0.2 mM ATP, pH 7.6) or phosphate-buffered saline (PBS), pH 7.4. Antibody binding to adsorbed actin was salt-independent in the range of concentrations used in these experiments. The amount of bound antibody was measured by using the alkaline phosphatase colorimetric assay. The optical density at 405 nm was monitored with a Molecular Devices (Menlo Park, CA) V_{max} microplate reader. Western blots of SDS gels were performed as described by Bulinski *et al.* (1983).

Cleavage of Actin by Thrombin and Hydroxylamine. Actin was cleaved with thrombin following the method of Mornet and Ue (1984). Hydroxylamine cleavage of actin was performed according to Sutoh (1981) with the following modification: hydroxylamine (2 M) was added to F-actin (1 mg/mL), and the reaction mixture was incubated overnight at room temperature (23 °C). The extent of actin cleavage was monitored by densitometry of SDS–polyacrylamide gels.

Actin-Activated ATPase Measurements. Actin-activated MgATPase activities of S-1 were measured in the presence and absence of 18–29 antibodies, as previously described (Reisler, 1980; DasGupta & Reisler, 1989). The ATPase measurements were carried out in the presence of 3 mM MgATP, 10 mM KCl, and 10 mM imidazole at pH 7.0 at 25 and 5 °C. Protein concentrations used for the 25 °C assays were as follows: actin, 3 μM; S-1, 3 μM; IgG, 0–15 μM; Fab, 0–27 μM. Protein concentrations used at 5 °C were as follows: actin, 3 μM; S-1, 25 μM; IgG, 0–12 μM.

Airfuge Binding Measurements. Rigor binding of S-1 (3 μM) to F-actin (3 μM) in the presence of antibody (IgG, 0–24 μM) was determined following the incubation of the protein mixtures under low-salt conditions (10 mM KCl/10 mM imidazole, pH 7.0) for 20 min at 25 °C. These samples were then spun for 20 min at 140000*g* in an air-driven ultracentrifuge (Beckman Instruments). Pelleted proteins were resuspended in the original solvent. Supernatant and resolubilized pellet fractions were denatured and run on 10 or 15% polyacrylamide gels (Laemmli, 1970). Densitometric scans of Coomassie Blue R-stained gels were analyzed to determine molar ratios of the S-1 and antibody pelleted with actin. Binding of S-1 to actin in the presence of ATP was measured as described for rigor conditions with the following modifications: For the 25 °C assays, protein mixtures containing F-actin (3 μM), S-1 (3 μM), and IgG (0–9 μM) were incubated for 20 min at 25 °C. MgATPγS (3 mM) was added to these solutions prior to their centrifugation. The ATP analog was used in lieu of ATP at 25 °C because of the rapid ATP hydrolysis at this temperature. For the 5 °C assays, the protein mixtures containing F-actin (3 μM), S-1 (25 μM), and IgG (0–12 μM) were equilibrated at 5 °C. MgATP (3 mM) was added just prior to centrifugation. The centrifugations were conducted at 5 °C, and the total ATP hydrolysis time in these experiments did not exceed 15 min (including ATP addition time). During this time, less than 50% of the ATP was hydrolyzed.

Concentration Determinations. Spectrophotometric determinations of protein concentrations at 280 nm were made by using the following extinction coefficients: S-1, $E^{1\%} = 7.50\ cm^{-1}$; actin, $E^{1\%} = 11.0\ cm^{-1}$; IgG, $E^{1\%} = 15.0\ cm^{-1}$; Fab, $E^{1\%} = 16.0\ cm^{-1}$; papain, $E^{1\%} = 25.0\ cm^{-1}$.

RESULTS

Characterization of the 18–29 Peptide Antibody. Enzyme-linked immunosorbent assays (ELISA) were used to determine the reactivity of the 18–29 peptide antisera toward actin. The antisera reacted with either G- or F-actin adsorbed to microtiter plates, exhibiting half-maximum reactivity with serum dilutions of about 1:2000. Preimmune controls showed

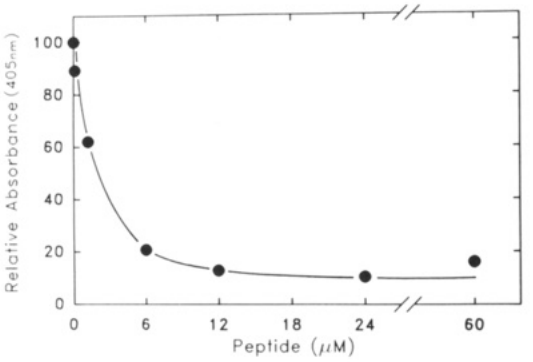

FIGURE 1: ELISA competition between the 18–29 peptide and G-actin. Anti-(18–29) actin peptide antiserum (diluted 1:250) was reacted in triplicate with G-actin adsorbed to microtiter plates (10 μg/well). Actin peptide (0–60 μM) was added simultaneously with the antiserum. Each data point represents OD_{405nm} readings after correction for background values in the absence of primary antibody. The maximum error margin between three averaged data sets was less than 5%. 100% absorbance at 405 nm corresponds to an OD reading of 0.80.

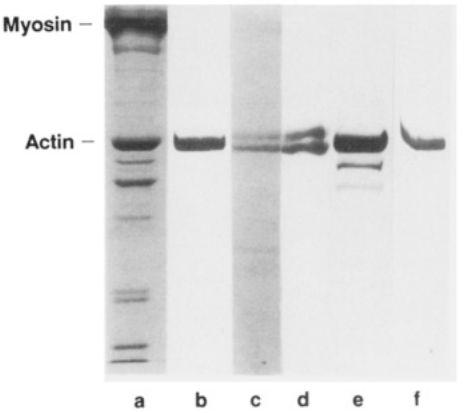

FIGURE 2: Immunoblots of electrotransferred SDS gels showing the reactivity of IgG(18–29) with myofibril extracts, and hydroxylamine- and thrombin-cleaved actins. Duplicate 10% SDS–polyacrylamide gels were either Coomassie-stained (lanes a, c, and e) or electrotransferred to nitrocellulose and immunoblotted (lanes b, d, and f). Lane a contains 10 μg of rabbit myofibril extract. Myosin heavy chain and actin bands are labeled. Lane b is the corresponding myofibril immunoblot reacted with 50 μg/mL 18–29 IgG. Lane c contains 5 μg of hydroxylamine-cleaved actin. The two main bands correspond to intact actin (upper band in the doublet) and actin fragment 13–375 [lower band in the doublet; reported by Sutoh (1982) to migrate at 41 kDa]. Lane d shows the corresponding immunoblot. Lanes e and f are the Coomassie-stained gel and corresponding immunoblot of 5 μg of thrombin-cleaved actin. The two main bands migrating below the intact actin correspond to the thrombin-cleaved actin fragments 29–375 and 114–375, reported to migrate at 37.5 and 27 kDa, respectively (Mornet & Ue, 1984). The immunoblots of cleaved actin were reacted with 50 μg/mL IgG(18–29).

no significant antibody binding to actin (data not shown). The specificity of the antibody for the amino-terminal (18–29) region of actin was tested in competitive ELISA experiments. As shown in Figure 1, the 18–29 actin peptide competed efficiently with G-actin for the binding to antibody. In separate ELISA titrations of IgG with G- and F-actin, the higher antibody reactivity seen with polymerized actin was consistent with the bivalent nature of IgG (data not shown). These results also verified that the 18–29 site is accessible to IgG in the actin filaments.

Cross-reactivity of the 18–29 antibody with other muscle proteins was tested by Western blot analysis of myofibrils. The affinity-purified IgG binds only to the actin band of myofibrillar proteins electrophoretically transferred to nitrocellulose from SDS–polyacrylamide gels (Figure 2, lanes a and b). Duplicate blots stained with amido black verified that all protein bands present in the Coomassie-stained gels were transferred and retained on the nitrocellulose membrane (data not shown).

The antibody binding site on actin was mapped to the 13–29 region by using two agents to cleave actin: the chemical hydroxylamine, which has been shown to cleave between Asn-12 and Gly-13 (Sutoh, 1982; Sutoh & Mabuchi, 1986), and the proteolytic enzyme thrombin, which cleaves between Arg-28 and Ala-29 (Muszbek et al., 1975; Mornet & Ue, 1984). Of the two hydroxylamine-cleaved products of actin, only the large 13–375 fragment was retained by our gel system. Thus, the two bands shown in Figure 2c correspond to this fragment and the uncleaved actin. Western blots of electrotransferred SDS–polyacrylamide gels showed that the antibody bound both to the intact and to the truncated actin (Figure 2d).

The proteolytic cleavage of actin by thrombin occurs between Arg-28 and Ala-29, Arg-39 and His-40, and Lys-113 and Ala-114 (Muszbek *et al.*, 1975). We selected short (10–15 min) digestion times that favored limited cleavage of actin, primarily at the Arg-28/Ala-29 site. The digestion products were identified according to their electrophoretic migration on SDS gels as compared to the mobility of protein standards. The 1–28 fragment was not retained by our gel system. The loss of antibody binding to thrombin-cleaved fragments of actin, particularly the 29–375 fragment (Figure 2f), localized the binding site to the first 29 residues and to no other epitope on the actin molecule. The retention of antibody binding by the hydroxylamine-truncated actin demonstrates that the 18–29 peptide antibody binds within the 13–29 amino-terminal region of actin.

Effect of 18–29 Antibodies on Acto–S-1 Rigor Interactions. The main goal of the experiments with the 18–29 antibody has been to verify that the 18–29 sequence on actin is a major contact site for S-1 under rigor conditions (Mejean *et al.*, 1987) and to assess also the involvement of this sequence in weak actomyosin interactions. In competitive ELISA, in agreement with earlier studies (Mejean *et al.*, 1987), S-1 readily displaced 18–29-specific IgG from F-actin adsorbed to microtiter plates (data not shown). Rigor binding of S-1 to acto–antibody complexes was then measured in solution studies. Analysis of pelleted fractions from S-1 and IgG samples cosedimented with F-actin demonstrates that S-1 and IgG(18–29) do not compete for binding to actin (Figure 3A,B). S-1 binds to actin at stoichiometric 1:1 molar ratios irrespective of the absence or presence of IgG bound to actin (Figure 3B). An estimate of a 10^5 M^{-1} binding constant for IgG to actin indicates that the binding of antibody is by 1 or 2 orders of magnitude weaker than that of S-1 to actin under similar rigor conditions (Greene & Eisenberg, 1980). Thus, if there were a significant overlap between the 18–29 actin region and the S-1 rigor binding site, the less tightly bound IgG would have been displaced from actin by S-1. Such displacement has not been observed; IgG binds equally well to actin in the presence and absence of S-1 (Figure 3B).

Binding of S-1 and 18–29 Antibodies to Actin in the Presence of ATP. The effect of 18–29 antibodies on weak acto–S-1 interactions was evaluated by measuring the actin-activated ATPase activity of S-1 in the presence of antibodies and the S-1·ATP binding to actin–antibody complexes. Low-salt conditions (10 mM KCl/10 mM imidazole, pH 7) were used in both activity and binding assays in order to maximize S-1 binding to actin. At 25 °C, IgG readily inhibited the ATPase activity of acto–S-1. Figure 4 shows an 84% loss of

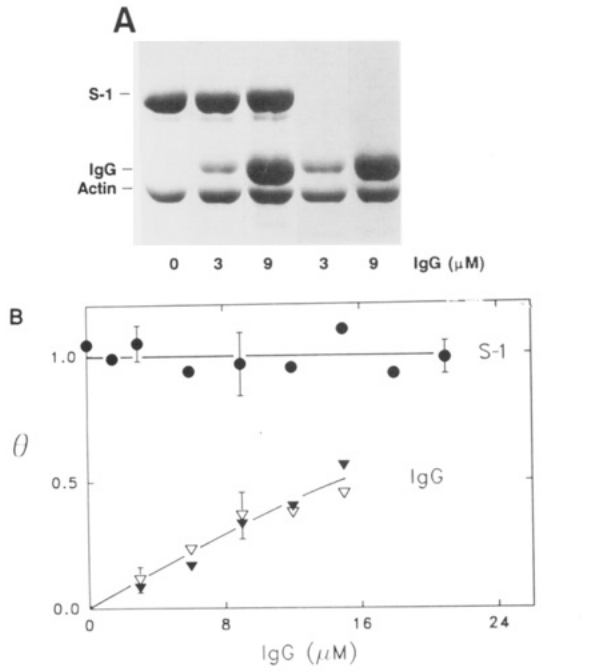

FIGURE 3: Rigor binding of S-1 to F-actin in the presence of IgG(18–29). The binding was measured in airfuge pelleting experiments as described under Materials and Methods. (A) Representative SDS–polyacrylamide gel (10%) showing the pelleted fractions of S-1 (3.0 μM) and IgG (0–9.0 μM) incubated with 3.0 μM F-actin. (B) Densitometric scans of Coomassie-stained gels similar to (A) were analyzed to determine the fractions of actin (θ) occupied by either S-1 or IgG. The fraction of actin bound to S-1 (●) was measured in the presence of IgG at concentrations ranging between 0 and 21 μM. The fraction of actin bound by IgG in the presence (△) and absence (▲) of 3.0 μM S-1 was measured with IgG at concentrations ranging between 0 and 15 μM. Since at least 95% of the actin addded was pelleted in these experiments, θ was calculated by assuming that 3.0 μM actin was present in each pelleted fraction. Error bars represent averages over several (between three and five) separate experimental sets.

activity when IgG is present at a 2:1 molar ratio relative to actin. This decrease in activity did not result only from S-1 displacement by IgG. As seen in a representative Coomassie-stained polyacrylamide gel (Figure 4, inset), increasing amounts of IgG(18–29) added to F-actin and S-1 in the presence of ATPγS indeed reduced the binding of S-1 to F-actin. However, densitometric analysis of such gels, shown in Figure 4, indicates that the antibody inhibits the acto–S-1 ATPase stronger than it does the binding of S-1·ATPγS to actin. In analogy to rigor conditions (Figure 3B), the binding of IgG to actin was not affected by the presence of S-1·ATPγS (data not shown).

Actin–IgG(18–29) complexes viewed by electron microscopy revealed that the bivalent antibody induced cross-linking of actin filaments (data not shown). Such cross-linking was strikingly reduced in the presence of S-1, thus accounting for the fact that rigor acto–S-1 binding was not disrupted by IgG (Figure 3). Yet since we were unsure whether actin bundling, if present, would impact the weaker acto–S-1 interactions, the acto–S-1 ATPase assays were repeated using the F_{ab}-(18–29) fragment of IgG. F_{ab} decreased acto–S-1 ATPase activity by more than 50% (Figure 5), demonstrating that the inhibition seen with IgG did not result from actin cross-linking or IgG size. Electron microscopy did not reveal any significant cross-linking of actin filaments by the F_{ab} fragment (data not shown). However, the much lower affinity of the monovalent F_{ab} for actin limited its use to a mere corroboration of the IgG experiments.

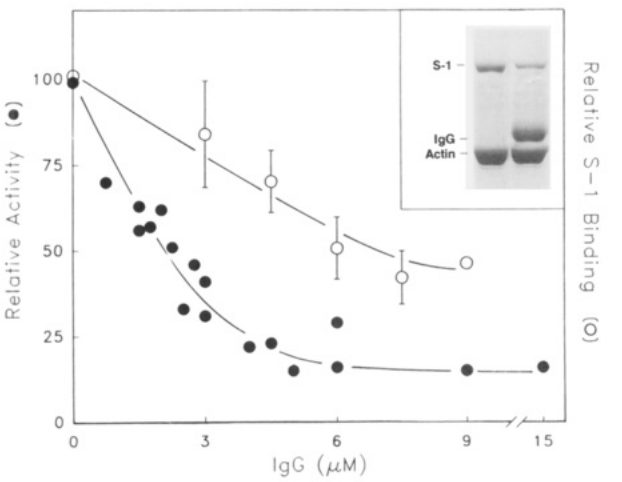

FIGURE 4: Comparison of actin-activated S-1 ATPase activities and S-1 binding to actin in the presence of IgG(18–29) at 25 °C. The ATPase activities (●) were measured in the presence in the presence of IgG, in a solvent containing 10 mM KCl, 10 mM imidazole, and 3 mM MgATP (pH 7.0). Activities were normalized to the rate of phosphate release in the absence of IgG; 100% activity = 0.32 μmol of phosphate/min. Protein concentrations were 3.0 μM actin, 3.0 μM S-1, and between 0 and 9.0 μM IgG. Similar results were obtained in three separate preparations of proteins. The S-1 bound to actin in the presence of IgG (○) was determined from densitometric analysis of SDS gels of pelleted proteins as described under Materials and Methods. In these experiments, actin (3.0 μM) was incubated with 3.0 μM S-1 in the presence of IgG (0–9.0 μM) in the same solvent as above except that 3 mM ATPγS was substituted for 3 mM ATP. S-1 binding to actin was normalized to the percent of actin bound to S-1 in the absence of IgG; 100% binding = 33% occupancy of actin by S-1. Error bars represent averages over at least three separate experimental data sets. The inset shows a representative Coomassie-stained gel of S-1·ATPγS pelleted with F-actin in the absence (lane 1) and presence (lane 2) of IgG. Protein concentrations were 3.0 μM actin, 3.0 μM S-1, and 7.5 μM IgG.

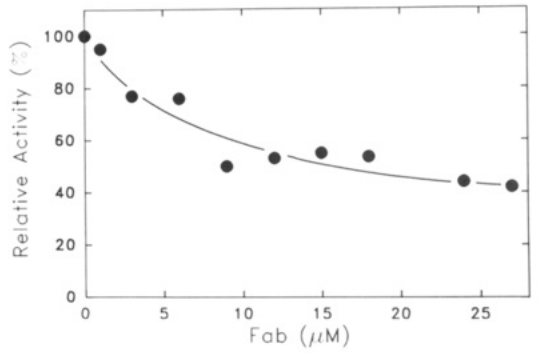

FIGURE 5: Effect of F_{ab}(18–29) on actin-activated S-1–MgATPase at 25 °C. The ATPase activities were measured in a solvent containing 10 mM KCl and 10 mM imidazole (pH 7.0). Protein concentrations in the assay mixture were 3.0 μM for actin and S-1 and between 0 and 24 μM for F_{ab}. ATPase activities were normalized to the rate of phosphate release in the absence of F_{ab}; 100% activity = 0.31 μmol of phosphate/min. Similar results were obtained in three separate preparations of proteins.

Possible temperature effects on the acto–S-1–antibody interactions were evaluated by repeating the ATPase and binding assays at 5 °C. Any potential differences in acto–S-1 binding due to the presence of ATP versus ATPγS were ruled out in control experiments. S-1 binding to actin at 5 °C was the same regardless of which nucleotide was used (data not shown). A significant difference in protein interactions between 25 and 5 °C was detected via the effect of antibody on acto–S-1 ATPase. Maximum inhibition of activity was achieved at a 2:1 IgG:actin molar ratio at 25 °C, while a 3:1 molar ratio of IgG to actin was required to achieve a somewhat

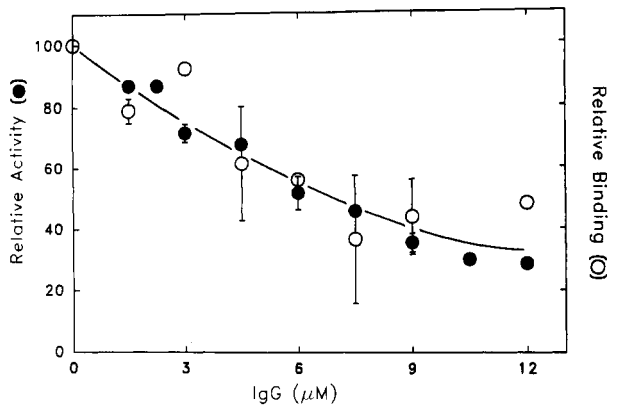

FIGURE 6: Comparison of actin-activated S-1 ATPase activity and S-1 binding to actin in the presence of IgG(18–29) at 5 °C. The ATPase activities (●) were measured in the presence of IgG, in a solvent containing 10 mM KCl, 10 mM imidazole, and 3 mM MgATP (pH 7.0). Actin (3.0 μM), S-1 (25 μM), and IgG (between 0 and 12 μM) were equilibrated at 5 °C prior to ATP addition. ATPase activities were normalized to the rate of phosphate release in the absence of IgG; 100% activity = 0.20 μmol of phosphate/min. Error bars reflect averages over at least three experimental sets. S-1 bound to actin (○) in the presence of IgG was determined from densitometric analysis of SDS gels containing the pelleted proteins (see Materials and Methods). Solvent conditions and protein concentrations were the same as described above for ATPase measurements at 5 °C. S-1 binding to actin was normalized to the percent of actin bound to S-1 in the absence of IgG; 100% binding = 62% occupancy of actin by S-1. Error bars represent averages over at least three separate experimental data sets.

weaker inhibition of acto–S-1 ATPase activity at 5 °C (Figure 6). These differences could not be attributed to temperature-dependent changes in antibody binding to actin; the amounts of IgG pelleted with actin were about the same at the two temperatures (data not shown). At 5 °C, the inhibition of acto–S-1 ATPase activity appears to be more closely correlated with the loss of S-1 binding to acto–IgG complexes than at 25 °C.

18–29 Antibodies Do Not Inhibit F_{ab}(1–7) Binding to Actin. The effects of IgG(18–29) on actomyosin interactions in the presence and absence of ATP are similar to those of F_{ab}(1–7) (DasGupta & Reisler, 1991, 1992). This similarity raised the question whether IgG(18–29) could modulate actin's function by perturbing its N-terminal residues 1–7. Cosedimentations of F_{ab}(1–7) with F-actin and IgG(18–29) (0–10 μM) did not reveal any change in F_{ab}(1–7) binding to actin (1.0 ± 0.10 F_{ab}/actin) due to increasing amounts of IgG(18–29) bound to actin (up to 0.30 mol of IgG per mole of actin). However, the binding of IgG(18–29) to actin was reduced somewhat by the bound F_{ab}(1–7). These differences in the effects of IgG(18–29) and F_{ab}(1–7) on each other's binding to actin are probably related to the different affinities of these antibodies for actin. More importantly, the absence of any significant perturbation of F_{ab}(1–7) binding to actin by the bound IgG(18–29) suggests that the effects of the latter on acto–S-1 interactions probably do not result from indirect changes induced in the 1–7 region on actin.

DISCUSSION

The goal of this work was to examine the role of the actin 18–29 region in the strong and weak actomyosin interactions, i.e., in the absence (rigor) and presence of ATP. Earlier immunochemical (Mejean *et al.*, 1987) and NMR (Moir *et al.*, 1987) studies and experiments with N-terminal actin peptides (Van Eyk *et al.*, 1991) have implicated the 18–29 N-terminal site in rigor binding to myosin. In the X-ray crystal structure determined for the actin–DNase I complex (Kabsch *et al.*, 1990), actin residues 20–28 form a hydrophilic loop. The two acidic residues, Asp-24 and Asp-25, contained within this loop were proposed as a likely site of contact with S-1.

In this work, we have used antibodies against the 18–29 actin peptide as a probe for this region on actin. Although the same antibodies were used in our previous study on actin–caldesmon interactions (Adams *et al.*, 1990), their specificity was yet to be documented. The present mapping of the antibody epitope to the 13–29 actin sequence, along with the strong competition between the 18–29 peptide and actin for antibody binding, demonstrates that the antibody is indeed specific for the 18–29 actin sequence. Importantly, also, the 18–29 antibodies do not react with any myofibrillar proteins except for actin.

We have shown that in solution IgG does not compete with S-1 for binding to actin under rigor conditions. Given the much stronger binding of S-1 (10^6–10^7 M^{-1}; Greene & Eisenberg, 1980) than that of IgG (~10^5 M^{-1}) to actin, the lack of any significant IgG displacement even in the presence of 1 mol of S-1 bound to actin demonstrates that S-1 can bind to actin via sites other than the 18–29 sequence (Labbe *et al.*, 1990; Kabsch *et al.*, 1990; Reisler, 1993). The formation of ternary S-1–actin–IgG complexes (Figure 3B) suggests that under rigor conditions the 18–29 sequence is not a major acto–S-1 contact site. This is reminiscent of a similar conclusion about the 1–7 residues on actin (Mejean *et al.*, 1987; Miller *et al.*, 1987; Sutoh *et al.*, 1991), albeit some mutual inhibition of S-1 and 1–7 antibody binding to actin could be readily measured in solution (DasGupta & Reisler, 1991). Thus, to the extent that the actomyosin interface can be analyzed in terms of its individual components, the 18–29 sequence ranks below the 1–7 N-terminal residues in its overall contribution to rigor actomyosin interactions.

The antibody results, however, should not be taken to indicate that the 18–29 sequence on actin does not interact with S-1 in the binary acto–S-1 complex. Given the results of NMR (Moir *et al.*, 1987) and peptide studies (Van Eyk *et al.*, 1991), such a contact may exist in the rigor acto–S-1 complex. Earlier ELISA results (Mejean *et al.*, 1987), which have been confirmed in this work, also appear to support this possibility. The solid-phase assays may be more sensitive than the solution experiments for probing the weak competition between IgG(18–29) and S-1 for actin. Yet, on a cautious note, the binding competition observed in ELISA may result from geometric constraints on the accessibility of closely packed actin molecules to both S-1 and IgG(18–29). The loss of the putative 18–29 actin contact with S-1, due to IgG(18–29) or F_{ab}(18–29) binding to actin, could be perhaps compensated by rearrangements along the multiple-contact acto–S-1 interface (Labbe et al., 1990). Such rearrangements can involve the strengthening of other existing contacts or the addition of a new contact site. These alterations need not be large and may result from an intrinsic capacity of the acto–S-1 interface for adjustments and transitions during the ATPase cycle.

The strong effect of 18–29 antibodies on acto–S-1 interactions in the presence of ATP is analogous to the inhibition of these interactions by F_{ab}(1–7) (DasGupta & Reisler, 1989, 1992). Yet, it appears unlikely that the 18–29 antibodies exert their effect by indirectly changing the 1–7 sequence on actin. The fact that the binding of IgG(18–29) to actin does not alter the binding of F_{ab}(1–7) to actin argues against any significant structural coupling between the 1–7 and 18–29 regions on actin.

An important feature of the 18–29 antibodies is that they inhibit acto–S-1 ATPase activity at 25 °C stronger than they do the binding of S-1·ATPγS to actin. Similar observations were also made with respect to the 1-7 sequence on actin (DasGupta & Reisler, 1992; Sutoh *et al.*, 1991). Thus, it appears that the requirements for the formation of a catalytically functional acto–S-1 complex are more stringent than indicated by the binding experiments alone. Interestingly, IgG(18–29) is a less potent inhibitor of acto–S-1 ATPase at 5 °C than at 25 °C (Figures 4 and 6). This is in contrast to F_{ab}(1–7) which inhibits the acto–S-1 ATPase equally well at both temperatures (DasGupta & Reisler, 1989). At present, it is not clear whether the modulation of actin by IgG(18–29) depends on temperature or, perhaps, the antibodies report on temperature-dependent changes in acto–S-1 interactions. Although these issues must await future clarification, our results and the work with actin mutants (Johara *et al.*, 1993) demonstrate clearly the important role of the 18–29 sequence on actin in weak actomyosin interactions.

The pronounced inhibition by IgG(18–29) of S-1 binding to actin in the presence but not in the absence of ATP can be explained by at least two simple scenarios. One possibility is that the above difference is linked to the relative strength of rigor and weak acto–S-1 interactions. A severalfold decrease in the affinity of S-1 for actin under rigor conditions could easily escape detection while a similar decrease in the presence of ATP would result in large shifts in the binding equilibrium between actin and S-1. Alternatively, the different effects of 18–29 antibodies on rigor and weak acto–S-1 interactions may reflect actual differences between acto–S-1 interfaces in the presence and absence of ATP. The size of IgG, and even that of F_{ab}(18–29), precludes a distinction between direct (blocking the 18–29 site), steric, and conformational effects of antibodies on acto-S-1 interactions in the presence of ATP. Thus, it is important that very similar observations were made with *Dictyostelium* mutants of actin in which Asp-24 and Asp-25 residues were replaced with histidines (Johara *et al.*, 1993). These mutants could bind to S-1 under rigor conditions but were unable to activate the myosin ATPase activity. The mutant results add credence to our interpretation of the antibody effects that the 18–29 sequence on actin plays an important role, either direct or indirect, in weak acto–S-1 interactions.

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