Interaction of skeletal-muscle myosin subfragment-1 with the actin-(338–348) peptide

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The data presented here confirm and provide further experimental evidence that rabbit skeletal-muscle myosin subfragment-1 (S-1) binds to the postulated actin-(338–348) hydrophobic segment [Kabsch, Mannherz, Suck, Pai and Holmes (1990) *Nature* (London) **347**, 37–44] with high affinity in the absence and presence of MgATP. The apparent dissociation constant of the S-1 interaction (5.5×10^{-7} M) with the actin-(338–348) peptide was of the same order of magnitude as that of the actin-(18–28) binding site (2×10^{-6} M). In similar conditions, fragmented (27 kDa–50 kDa–20 kDa) S-1 also bound to the peptide. Antibodies directed to the vicinal sequence 348–358 were rapidly eliminated from actin by S-1 interaction and weakened S-1 binding to monomeric or filamentous actin. The antigenic site

(348–358) is located very close to the C-terminal S-1-binding site (360–369) and encompasses some residues (Leu-349 and Phe-352) included in the hydrophobic S-1-binding region [Schröder, Manstein, Jahn, Holden, Rayment, Holmes and Spudich (1993) *Nature* (London) **364**, 171–174]. It was observed that anti-[actin-(348–358)] antibodies were also unable to decrease actomyosin ATPase activity, in contrast with previous results obtained with anti-[actin-(18–28)] antibodies [Adams and Reisler (1993) *Biochemistry* **32**, 5051–5056]. The hydrophobic actin-(338–348) peptide used in considerable excess was unable to perturb acto-S-1 and S-1 activities in contrast with results obtained with the N-terminal actin peptide [Kögler, Moir, Trayer and Ruegg (1991) *FEBS Lett.* **294**, 31–34].

INTRODUCTION

Many direct observations and indirect experimental approaches have pinpointed several segments in actin subdomain-1 which bind to skeletal-muscle myosin subfragment-1 (S-1) and thus belong to the actomyosin interface.

A multisite interface model (Labbé et al., 1990) has also been proposed to take account of myosin head binding to the N-terminal 1–28 segment (Méjean et al., 1987; Kögler et al., 1991) and to the 360–372 terminal region (Labbé et al., 1992). This last sequence could be restricted to 360–369 (Crosbie et al., 1992). Finally, two limited actin segments encompassing residues 96–103 and 112–125 have been identified as myosin head-binding sites (Labbé et al., 1993), and Arg-95 was shown to cross-link to S-1 (Bonafé et al., 1993). The spatial proximity of several residues (i.e. Phe-124 and Tyr-362) in two critical actin segments (Hennessey et al., 1993) clearly illustrates the continuity of the interface which also includes residues in sequence 77–95 (Smith et al., 1993). There seems to be discontinuity in the interaction in the presence of MgATP. It was thus recently reported that the actin-(77–95) peptide binds equally well to S-1 and to S-1-ATP (Smith et al., 1993), but the affinity of the N-terminal binding region (sequence 1–28) for S-1 decreases in the presence of MgATP (Kögler et al., 1991).

On the basis of affinity (Eisenberg and Greene, 1980), three types of S-1-actin complex can be defined: (a) a rigor complex in which there is no occupant at the ATP-binding site and which has the highest stability; (b) a strong binding complex with the ATP-binding site occupied by MgADP or its analogues and which has moderate stability; (c) a weak binding complex with the ATP-binding site occupied by MgATP or its analogues and which has low stability.

The occurrence of hydrophobic interactions in the actin-myosin complex has previously been suggested (Chalovich et al., 1983; Katoh et al., 1984; Kabsch et al., 1990), but no fragments known to be binding structures are hydrophobic, but rather are hydrophilic with charged clusters. Two accessible hydrophobic residues (Ile-345, Leu-346) in actin have been proposed as being involved in the S-1-binding region (Kabsch et al., 1990), and this was confirmed by Schröder et al. (1993) during the preparation of the present paper.

A previous observation (Labbé et al., 1992) indicated that polyclonal antibodies directed to the actin-(300–355) fragment were excluded from actin in the presence of S-1, and the above hypothesis (Schröder et al., 1993) led us to investigate the hydrophobic α -helix (338–348) as a possible S-1-binding site. Our present results indicate that S-1 binds to this segment in the absence or presence of MgATP. No inhibitory properties towards acto-MgATPase were observed in the presence of a large excess of the related synthesized peptide.

MATERIALS AND METHODS

Chemicals

α -Chymotrypsin was purchased from Worthington. Thrombin from human plasma, trypsin (treated with *N*-tosyl-L-phenylalaninechloromethane), gelatin and crude pancreatic-digested gelatin were from Sigma Chemical Co. CNBr-activated Sepharose 4B was obtained from Pharmacia.

Anti-(sheep IgG) and anti-(rat IgG) antibodies labelled with alkaline phosphatase were from Biosys (Compiègne, France).

All other chemicals were of analytical grade.

Abbreviations used: S-1, rabbit skeletal-muscle myosin subfragment-1; F-actin, filamentous actin; G-actin, monomeric actin; anti-(285–375), antibodies against actin sequences including Met-305, Met-325 and Met-355; antibody population 1, antibodies against the actin sequence including Met-325; antibody population 2, antibodies against the actin sequences localized around Met-325 and Met-355.

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Proteins and peptides

Rabbit myosin and α -chymotryptic myosin S-1 were obtained from back muscle by the method of Weeds and Taylor (1975) and purified on Sephacryl S-200 equilibrated with 50 mM Tris/HCl, pH 7.5.

The trypsin derivative (27–50–20 kDa) S-1 was prepared as described by Mornet et al. (1979).

Rabbit skeletal-muscle G- and F-actins were purified as described by Eisenberg and Kielly (1974). G-actin was added to 2 mM Tris/HCl buffer, pH 7.6, containing 0.2 mM ATP and 0.1 M CaCl_2 . F-actin was added to 2 mM Tris/HCl buffer, pH 7.6, containing 2 mM MgCl_2 , 10 mM KCl and 0.2 mM CaCl_2 . S-Carboxymethylated actins were obtained as previously described (Hirs, 1967).

The synthesized peptide corresponding to the 338–348 amino acid sequence of actin was obtained by solid-phase synthesis using the 9-fluorenylmethyloxycarbonyl-*t*-butyl system with a Milligen pepsynthetizer TM 9050 (Milligen Bioresearch Division, Watford, Herts., U.K.). Lysine was added at the N- and C-terminal portions of the 338–348 peptide. The peptide was tested by analytical h.p.l.c. and amino acid analysis.

The purity of proteins and protein fragments was checked by SDS/PAGE (Laemmli, 1970) on 5–18% gradient polyacrylamide slab gels. The protein bands were located after Coomassie Blue staining, and the protein concentrations determined spectrophotometrically (Labbé et al., 1990).

Protein concentrations were determined spectrometrically with an absorption coefficient of $A_{280\text{nm}}^{1\%} = 7.5\text{ cm}^{-1}$ for S-1, 11 cm^{-1} for F-actin and 15 cm^{-1} for the antibodies. The absorption coefficient of $A_{290\text{nm}}^{1\%}$ for actin was 6.4 cm^{-1} .

Immunological techniques

Polyclonal antibodies specifically directed against the 285–375 amino acid sequence of actin were obtained from sheep antisera, and their specificity determined as previously reported (Boyer et al., 1987; Houmeida, 1991). These antibodies were specific to three epitopes in skeletal-muscle actin, including residues Met-305, Met-325 and Met-355. The different antibody populations were selectively purified by affinity chromatography using scallop actin, which differs from rabbit actin by a mutation at position 306 (Boyer et al., 1987; Hué et al., 1989), immobilized on Sepharose 4B. Anti-[actin-(285–375)] antibodies were first purified on a rabbit S-carboxymethylated skeletal-muscle actin column. Related populations directed to epitopes near Met-325 and Met-355 were retained on a scallop S-carboxymethylated actin column (population 2); the antibodies specific to the epitope including Met-305 were not retained and discarded. Another anti-actin antibody population (population 1) extracted (Houmeida, 1991) from anti-(oxidized actin) immunoserum (Benjamin et al., 1986) was demonstrated to be directed against the 305–326 amino acid sequence. Two antibody populations directed against epitopes in sequence 305–326 (population 1) and sequence 325–359 (population 2) were thus used in this study.

Antibodies directed against S-1 were raised in rats as described by Robin et al. (1976) and induced by the N-terminal 27 kDa segment of the rabbit S-1 tryptic derivative.

Direct and competitive e.l.i.s.a.s were used to monitor interactions between proteins as described by Méjean et al. (1987) and Labbé et al. (1990). G-actin (1 $\mu\text{g/ml}$), F-actin (0.1 mg/ml) or synthesized actin-(338–348) peptide (10 $\mu\text{g/ml}$) in 15 mM Na_2CO_3 /35 mM NaHCO_3 buffer, pH 9.5, were coated (150 μl) on microtitre plate wells (Nunc) and incubated overnight at 4 °C. After being washed in PBS buffer containing Tween 20 (0.1%),

excess protein or peptide was discarded and the wells were saturated with gelatin (0.5%) and gelatin hydrolysate (3%, w/v) or lactalbumin (3%, w/v). Various antibody concentrations were then incubated in the absence or presence of F-actin (saturation concentration) for 1 h at 37 °C. Each assay was performed in triplicate. Control assays were carried out in wells saturated with gelatin and gelatin hydrolysate only. We observed no non-specific antibody binding. G-actin was coated at lower than critical concentration (1 $\mu\text{g/ml}$) to prevent polymerization.

ATPase assay

Actin-activated Mg^{2+} -dependent ATPase activity of S-1 was determined at 25 °C for 45 min in the presence of 50 mM Tris/HCl buffer, pH 8.0, containing 10 mM KCl, 5 mM ATP and 5 mM MgCl_2 . The activity was measured in the presence of increasing antibody population 2 concentration (0–80 $\mu\text{g/ml}$) for various actin concentrations (2–20 μM), and S-1 was added at 0.4 μM . The activity was also measured in the presence of increasing concentration of synthesized actin-(338–348) peptide (0–1 mg/ml), F-actin (12 μM) and S-1 (0.4 μM). Phosphate release was determined as described by Labbé et al. (1982).

Figure 1 Reactivity of antibody population 1 (a) and antibody population 2 (b) towards G-actin and F-actin in the presence of S-1

(a) Antibody population 1 (10 $\mu\text{g/ml}$) was allowed to react with G-actin (●) or F-actin (□) in the presence of increasing S-1 concentrations (0–300 $\mu\text{g/ml}$). (b) Antibody population 2 (10 $\mu\text{g/ml}$) was allowed to react with G-actin (●) or F-actin (□) in the presence of increasing S-1 concentrations (0–200 $\mu\text{g/ml}$). The binding of specific anti-actin antibodies was monitored at 405 nm by e.l.i.s.a. as described in the Materials and methods section.

Figure 2 Effect of the presence of S-1 on the reactivity of antibody population 2 towards G-actin (a) and F-actin (b) in the presence of MgATP

(a) Antibodies (5 $\mu\text{g/ml}$) were allowed to react in the presence of increasing S-1 concentrations (0–1 mg/ml) with G-actin in the absence ($\triangle$) or presence ($\blacktriangle$) of 5 mM MgATP. (b) Antibodies (5 $\mu\text{g/ml}$) were allowed to react in the presence of increasing S-1 concentrations (0–1 mg/ml) with F-actin in the absence ($\triangle$) or presence ($\blacktriangle$) of 5 mM MgATP. The binding of specific anti-actin antibodies was monitored at 405 nm by e.l.i.s.a as described in the Materials and methods section.

RESULTS

Immunochemical evidence for the involvement of the 325–360 sequence of actin in the actin–S-1 interface

In a previous analysis (Labbé et al., 1992), we observed that, at saturating S-1 concentrations, the apparent dissociation constant (K_d) of anti-[actin-(285–375)] antibodies varied from 3×10^{-8} to 6×10^{-8} M in the absence of S-1 to 1×10^{-6} M in the presence of S-1. In contrast, S-1 binding was only very slightly affected (less than 2-fold) in the presence of antibodies. Similar results were obtained with S-1(A1) and S-1(A2) isoforms (Labbé et al., 1992).

The apparent dissociation constants of purified antibody populations (1 and 2) to G- or F-actin coated on the plastic of microtitre plates were of the same order (1×10^{-7} to 5×10^{-7} M), and it can be assumed that the related antigenic epitopes were accessible in G-actin and F-actin.

The present data show that, in the presence of increasing S-1 concentrations (0–1 mg/ml), antibody populations 1 and 2 were partially prevented from interacting with G- and F-actin in the absence (Figures 1a and 1b) or presence (Figures 2a and 2b) of 5 mM MgATP.

In the reciprocal experiment, the effect of antibody binding on S-1 interaction with actin in conditions of rigor was examined.

Figure 3 Binding of rabbit skeletal-muscle 1 to G- and F-actin in the presence of antibodies

G-actin ($\bullet$) and F-actin ($\square$) were coated on microtitre plates (1 $\mu\text{g/ml}$ and 100 $\mu\text{g/ml}$ respectively). Rabbit S-1 was added at 15 $\mu\text{g/ml}$ (a) in the presence of antibody population 1 at increasing concentration (0–400 $\mu\text{g/ml}$) and (b) in the presence of antibody population 2 at increasing concentration (0–160 $\mu\text{g/ml}$).

S-1 interaction was not affected at all by binding of antibody population 1 (Figure 3a); in contrast, it was partially affected by binding of population 2 (Figure 3b). Similar results were obtained when G- and F-actin were studied. These results suggest that the subdomain 3 antigenic sequence around residue 325 is not implicated in the actin–S-1 interface but undergoes conformational changes. In contrast, the subdomain-1 antigenic sequence near Met-355 seems to be subject to a more direct steric and reciprocal effect between S-1 and IgG antibody population 2, but the proximity of the previously reported S-1-binding site on the 360–372 sequence (Labbé et al., 1992) could explain this effect.

Direct binding of S-1 or tryptic S-1 to actin-(338–348)

The hydrophobic component of the acto–S-1 interaction has been suggested to be centred around the 341–349 α -helix of actin (Kabsch et al., 1990), including the exposed hydrophobic residues 345 and 346. In addition, this sequence is, like amino acids 18–28, highly conserved throughout evolution. Thus we investigated S-1 binding to the synthesized helical peptide actin-(338–348), supplemented with a lysine residue at the N- and C-termini to increase its solubility.

When the synthesized actin-(338–348) peptide was coated on microtitre plates, we observed that S-1 was able to bind at high

Figure 4 Binding of (a) rabbit skeletal-muscle S-1 and (b) rabbit tryptic S-1 to actin-(338–348)

Actin-(338–348) was coated (10 $\mu\text{g/ml}$) on microtitre plates and (a) S-1 was added at increasing concentrations (0–1 mg/ml) in the absence (Δ) or presence ($\blacktriangle$) of 5 mM MgATP, or (b) rabbit (27–50–20 kDa) S-1 was added at increasing concentrations (0–1 mg/ml). S-1 binding was followed by anti-(27 kDa N-terminal S-1 fragment) antibodies.

Figure 5 Reactivity of anti-IAEDANS antibodies towards IAEDANS-labelled G-actin or IAEDANS-labelled actin-(355–375) in the presence of S-1

Anti-IAEDANS antibodies (0.2 $\mu\text{g/ml}$) were allowed to react with IAEDANS-labelled G-actin ($\bullet$) or IAEDANS-labelled actin-(355–375) ($\circ$) in the presence of increasing concentrations (0–2 mg/ml) of rabbit skeletal-muscle S-1 (A1,A2).

affinity ($5.5 \times 10^{-7} \pm 0.5 \times 10^{-7}$ M) in the absence of 5 mM MgATP. This interaction still occurred in the presence of 5 mM MgATP (Figure 4a).

We observed (Figure 4b) that tryptic S-1 was also able to bind to the coated peptide. We have previously shown (Labbé et al., 1993) that, under similar conditions, cleaved S-1 binds to the actin-(360–372) peptide but not to the 96–132 amino acid sequence.

Effect of antibody population 2 and actin-(338–348) peptide on acto-S-1 MgATPase activity

E.I.s.a. showed the absence of reactivity between antibody population 2 and actin-(338–348) peptide (results not shown).

We have previously demonstrated (Boyer et al., 1987) that the epitope including Met-355 was limited in its C-terminal part by residue 358. This indicates that an epitopic sequence could be located between residue 348 and residue 358.

Acto-S-1 MgATPase activity was measured in the presence of increased antibody population 2 concentrations, and the experiments were repeated with various actin concentrations. No effect was detected, in spite of the localization of these antibodies between the two S-1-binding sites (amino acid sequences 360–372 and 338–348) and their high affinity for actin ($K_d = 1.5 \times 10^{-7}$ M).

In the presence of actin-(338–348) (0–1 mg), the acto-S-1 MgATPase activity was not modified and in the absence of F-actin and basal MgATPase of S-1 was not affected (results not shown).

Reactivity of anti-IAEDANS antibodies towards IAEDANS-labelled residue 374 of actin, in the presence of S-1

Finally it was previously observed that binding of anti-IAEDANS antibodies ($K_d = 6 \times 10^{-9}$ M) to IAEDANS-modified Cys-374 was substantially weakened after S-1 binding to F-actin (Méjean et al., 1987) or G-actin (Figure 5). When we used the IAEDANS-modified actin-(355–375) peptide (CB9), which binds to S-1 (Labbé et al., 1992), the affinity of anti-IAEDANS antibody was not affected in the presence of S-1 (Figure 5). The location of Cys-374 in a proximal position to the 338–348 amino acid segment of actin (McLaughlin et al., 1993) close to S-1 could easily explain these observations. In connection with this, it has been demonstrated that S-1 protects the actin C-terminus from tryptic cleavage (Crosbie et al., 1992).

DISCUSSION

The present data confirm and provide further experimental evidence that rabbit skeletal-muscle S-1 binds to the postulated actin 338–348 hydrophobic segment (Kabsch et al., 1990) with high affinity in both the absence and presence of MgATP. Under similar conditions, trypsin-cleaved S-1 also binds to the peptide. We thus used an antibody population ($K_d = 6 \times 10^{-7}$ M) directed against the conserved vicinal (348–358) sequence (population 2) that flanks the suspected region.

Antibody population 2 was rapidly eliminated from actin by interaction with S-1, and, in the reciprocal experiment, the antibodies weakened S-1 binding to monomeric and filamentous actin. The antigenic site (348–358) is located very close to each S-1 binding site on actin, i.e. the C-terminal sequence (360–369) encompassing some hydrophobic residues (Leu-349 and Phe-352) and the α -helix-containing sequence (338–348).

We also observed that direct binding between the synthesized peptide actin-(338–348) and S-1 was not dependent on MgATP (5 mM).

According to Schröder et al. (1993), the interaction between myosin and actin reveals some regions of close contact. One region of contact involves a charged lysine-rich loop (amino acids 624–647) of myosin and the negative cluster, including

carboxy residues 1, 2, 3, 4, 24, 25, 99 and 100. This region of contact is electrostatic and could be involved in weak cross-bridge binding. It has been demonstrated by cross-linking studies that, in the presence of ATP, the N-terminal acidic sequence of actin interacts infrequently and only at restricted sites of the lysine-rich sequence, in contrast with the rigor state (Yamamoto, 1989). A second site of contact involves the exposed hydrophobic α -helix at residues 338–348. This interaction has been analysed in the present study and appears to be independent of the presence of ATP. So this site is a possible high-affinity contact site in the presence of nucleotide and may be implicated in the weak interaction.

The apparent dissociation constant (5.5×10^{-7} M) of the S-1 interaction with actin-(338–348) was similar to that of the actin-(18–28)-binding site (2×10^{-6} M). In contrast, the N-terminal site, which is strongly hydrophilic, appeared to be implicated in actomyosin MgATPase activity (Kôgler et al., 1991). In this connection, cleaved S-1 was shown to interact with the synthesized actin-(338–348) peptide with a similar affinity to that of the uncleaved S-1 in the presence of MgATP. As cleaved S-1 is unable to interact with the hydrophilic binding site (residues 96–103) (Labbé et al., 1993), and loses its ability to be activated by F-actin, but recovers this property after chemical cross-linking (Labbé et al., 1982), this hydrophobic S-1-binding site on the 338–348 sequence of actin did not by itself seem to be implicated in the actin-stimulated S-1 MgATPase activity. Two experiments could support this interpretation. First, antibodies directed to the actin sequence (348–358) close to the hydrophobic helix did not affect actomyosin ATPase activity, in contrast with previous results obtained with anti-[actin-(18–28)] antibodies (Adams and Reisler, 1993). Secondly, the hydrophobic peptide actin-(338–348) alone when used in considerable excess was unable to perturb acto-S-1 and S-1 activities, in contrast with results (Kôgler et al., 1991) obtained with the N-terminal actin peptides.

The α -helical peptide actin-(338–348) was synthesized with only one lysine at each extremity (to improve its solubility) instead of acidic residues which would enhance the solubility but could mimic the charged actin cluster and thus perturb the ionic interaction in the actomyosin interface.

The binding of S-1 to the synthesized actin-(338–348) peptide could explain the weakening of anti-IAEDANS antibody binding to Cys-374-labelled actin. The location of this residue in the proximity of the 338–348 sequence has been shown by McLaughlin et al. (1993).

The simultaneous accessibility of the two major sites (i.e. amino acids 18–28 and 338–348) in S-1-F-actin nucleotide complexes is currently being investigated.

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