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Characterization of an actin-myosin head interface in the 40–113 region of actin using specific antibodies as probes

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Evidence for the participation of the 1–7 and 18–28 *N*-terminal sequences of actin at different steps of actin–myosin interaction process is well documented in the literature. Cross-linking of the rigor complex between filamentous actin and skeletal-muscle myosin subfragment 1 was accomplished by the carboxy-group-directed zero-length protein cross-linker, 1-ethyl-3-[3-(dimethylamino)propyl]carbodi-imide. After chaotropic depolymerization and thrombin digestion, which cleaves only actin, the covalent complex with M_r 100000 was characterized by PAGE. The linkage was identified as being between myosin subfragment 1 (S-1) heavy chain and actin-(1–28)-peptide. The purified complex retained *in toto* its ability to combine reversibly with fresh filamentous actin, but showed a decrease in the $V_{max.}$ of actin-dependent Mg^{2+}-ATPase. By using e.l.i.s.a., S-1 was observed to bind to coated monomeric actin or its 1–226 *N*-terminal peptide. This interaction strongly interfered with the binding of antibodies directed against the 95–113 actin sequence. Moreover, S-1 was able to bind with coated purified actin-(40–113)-peptide. Finally, antibodies directed against the 18–28 and 95–113 actin sequence, which strongly interfered with S1 binding, were unable to compete with each other. These results suggest that two topologically independent regions are involved in the actin–myosin interface: one located in the conserved 18–28 sequence and the other near residues 95–113, including the variable residue at position 89. Other experiments support the 'multisite interface model', where the two actin sites could modulate each other during S-1 interaction.

INTRODUCTION

The interaction of actin with myosin and the associated thin-filament proteins as well as their cyclic interactions coupled with the hydrolysis of ATP are the main molecular events leading to muscle contraction.

Analysis of the rigor complex of muscle thin filaments decorated with myosin subfragment-1 (S-1), using low-resolution electron microscopy and image reconstruction, led to the development of a three-dimensional model of the acto–S-1 complex. In contrast with Amos *et al.* (1982), Toyoshima & Wakabayashi (1985) and Milligan & Flicker (1987) have suggested that the S-1-binding site covers an entire long face of the actin monomer.

Several studies using a variety of approaches have indicated a possible role for the *N*-terminal residues of skeletal actin in the acto-myosin interaction. According to Sutoh (1983), the same acidic *N*-terminal residues of the actin segment can be cross-linked by the zero-length cross-linker 1-ethyl-3-[3-(dimethylamino)propyl]carbodi-imide (EDC) to either the 20 kDa *C*-terminal or the 50 kDa central fragment of S-1 (Mornet *et al.*, 1981). The carboxy groups of actin involved in these cross-linked species have been located by Elzinga (1987) at positions 1–4 in the sequence. The interaction of the *C*-terminal segment of actin spanning residues 356–375 with the alkali light chain-1 of S-1 has also been described by Sutoh (1982) and Labbé *et al.* (1986).

Antibodies directed against actin have been obtained and characterized by Benyamin *et al.* (1986) and Roustan *et al.* (1986). Méjean *et al.* (1986, 1987), using an e.l.i.s.a. titration, have shown on filamentous actin that specific antibody populations directed against sequence 1–7 could still bind to the acto–S-1 complex and form a ternary complex. Such immunoassay results obtained on solid phase were confirmed by Miller *et al.* (1987), who studied the interaction in solution. The *N*-terminal acidic residues of actin are not essential for acto–S-1 rigor binding. However, Das Gupta & Reisler (1989*a*,*b*), showed, in the presence of ATP, a regulation of acto–S-1 interactions by specific anti-[actin-(1–7)-sequence] antibodies (1–7 antibodies).

Méjean *et al.* (1987), using a second antibody population directed against the 18–28 segment, characterized a major contact area for S-1 in the hydrophilic constant 18–28 sequence (Hue *et al.*, 1989). ^{1}H n.m.r. binding studies have further indicated the involvement of the 1–44 *N*-terminal segment in the recognition of S-1 heavy chain (Moir *et al.*, 1987). Synthetic actin-(1–28)-peptide is able to compete with filamentous (F-)actin for S-1 and to exert some control over S-1 activity (Van Eyk & Hodges, 1989).

Finally, Bertrand *et al.* (1988), using glutaraldehyde and *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ) to cross-link the rigor complex, has shown the formation of an S-1 heavy chain–actin peptide cross-linked complex, which differed from that obtained with EDC. By using a simple approach with specific anti-actin antibodies, the actin peptide has been identified by us as 1–28 in the case of EDC cross-linking and 1–28 and 40–113 for glutaraldehyde or EEDQ (Benyamin *et al.*, 1986; Bertrand *et al.*, 1988).

Analysis of the antigenic structure of actin using an antibody population specific for different parts of the actin sequence has revealed three independent regions in the *N*-terminal third of the actin monomer (Benyamin *et al.*, 1988). From this study it appears that antibodies directed against the 18–28 segment do

Abbreviations used: S-1, myosin subfragment 1; acto–S-1, acto-myosin S-1; actino-peptide-1, actin-(1–28)-peptide; EDC, 1-ethyl-3-[3-(dimethylamino)propyl]carbodi-imide; EEDQ, *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline; DTE, dithioerythritol; 95–113 antibodies, anti-[actin-(95–113)-sequence] antibodies; 18–28 antibodies, anti-[actin-(18–28)-sequence] antibodies; 1–7 antibodies, anti-[actin-(1–7)-sequence] antibodies; G-actin, monomeric actin; F-actin, filamentous actin.

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not compete with those directed against the 40–113 segment, which are specific for an epitope near residue 103, or with antibodies directed against the 1–7 *N*-terminal segment of monomeric actin.

On the basis of a 'multi-site interface model' we can postulate which part of the 40–113 segment is involved in the S-1 binding site and whether there is a close relation between the two S-1 binding sites, i.e. the 18–28 and 40–113 segments. These results have been reported previously in an abstract form (Labbé *et al.*, 1989).

MATERIALS AND METHODS

Chemicals

Chymotrypsin, thrombin from human plasma and trypsin [treated with tosylphenylalanylchloromethane ('TPCK')] were purchased from Worthington, Sigma and Serva respectively. Ultrogel ACA 54 was from IBF Biotechnics (Villeneuve la Garenne, France). EDC was a Sigma product. Anti-rabbit IgG, and anti-rat IgG antibodies labelled with peroxidase or alkaline phosphatase, were from Biosys (Compiègne, France).

All other chemicals were of analytical grade.

Preparation of proteins

Rabbit chymotryptic myosin S-1 was obtained by the method of Weeds & Taylor (1975) and purified over Sephacryl S-200, eluted with 50 mM-Tris/HCl buffer, pH 7.5.

Rabbit skeletal-muscle monomeric (G-) and F-actins were purified as described by Eisenberg & Kielly (1974). G-actin was in 2 mM-Tris/HCl buffer, pH 7.6, containing 0.2 mM-ATP and 0.1 mM-$CaCl_2$. F-actin was in 2 mM-Tris/HCl buffer (pH 7.6)/2 mM-$MgCl_2$/10 mM-KCl/0.2 mM-$CaCl_2$.

Protein concentrations were determined spectrometrically (Méjean *et al.*, 1986) with specific absorption coefficients ($A^{1\%}_{280}$) of 7.5 cm^{-1} for S-1, 17 cm^{-1} for the 40–113-residue peptide, 11 cm^{-1} for actin and 15 cm^{-1} for antibodies. The $A^{1\%}_{290}$ for G-actin was 6.4 cm^{-1}.

Cross-linking reactions and proteolytic cleavage of the covalent complexes

F-actin (2 mg/ml) and S-1 (2 mg/ml) were incubated with EDC (5 mM) in 100 mM-Mes, pH 6.0, for 120 min at 20 °C. At the end of the reaction, S-1 not cross-linked was released by adding an equal volume of dissociating solution (20 mM-sodium pyrophosphate/10 mM-$MgCl_2$/200 mM-KCl/100 mM-Hepes buffer, pH 8.5. After centrifugation at 160000 *g* for 60 min at 4 °C, the covalent complex and F-actin were recovered in the pellet. Residual S-1 was totally recovered in the supernatant (see Fig. 1 below). The pellet containing the covalent acto–S-1 complex was digested with thrombin after F-actin depolymerization as described by Labbé *et al.* (1986). The pellet was first incubated in 2 ml of depolymerizing solution (0.6 M-KI/5 mM-sodium thiosulphate/0.5 mM-dithioerythritol/0.1 mM-ATP/0.5 mM-$CaCl_2$/50 mM-Tris/HCl buffer, pH 7.5) for 15 min at 4 °C according to Rouayrenc *et al.* (1985). After centrifugation, at 160000 *g* for 60 min at 4 °C, the supernatant was dialysed overnight against 0.2 mM-dithioerythritol (DTE)/1 mM-EDTA/2 mM-Tris/HCl buffer, pH 8.0. Human plasma thrombin was then added and the mixture was incubated for 300 min at 25 °C. This enzyme is known to split EDTA–G-actin into fragments corresponding to residues 1–28, 29–39, 40–113 (peptide M), 114–375 (peptide L) and 40–375 (peptide K) (Muszbeck *et al.*, 1975), with the use of this procedure, thrombin has no effect on the heavy or alkali light chain in S-1 (Henry *et al.* 1985; Chaussepied *et al.*, 1986).

After dialysis and centrifugation at 160000 *g* for 60 min at 4 °C, the supernatant was shown to contain the S-1–actino-peptide-1 complex in which the actin-(1–28)-peptide (actino-peptide-1) is covalently linked to S-1 (Labbé *et al.*, 1986; Bertrand *et al.*, 1988) and the large actin thrombic peptides (K, L and M).

Concentration of the actino-peptide-1–S-1 complex was estimated by densitometric measurements on a Shimadzu model CS-930 high-resolution gel scanner equipped with a computerized integrator, with S-1 heavy chain as standard.

Actin cleavage

Preparation of the 40–113-residue actin derivative. The actin segment consisting of residues 40–113 was generated by thrombin cleavage of monomeric actin (2 mg/ml) with a ratio of 2 units/mg under conditions similar to those described by Muszbeck *et al.* (1975), except that the thrombic digest of actin was isolated by gel filtration over an Ultrogel ACA 54 column (2.4 cm × 150 cm) eluted with 10 mM-Tris/HCl buffer, pH 8.0. Its purity was assessed by gel electrophoresis. E.l.i.s.a. indicated no contamination with the thrombic peptide consisting of residues 1–28 or the *C*-terminal peptide (114–375).

Preparation of the 1–226-residue actin peptide. A freshly prepared sample of G-actin (6 mg/ml) was subjected to digestion by *Staphylococcus aureus* V8 proteinase as described by Roustan *et al.* (1985) for 4 min at room temperature. This large *N*-terminal fragment (1–226 sequence) was purified by gel filtration over an Ultrogel ACA 54 column (180 cm × 3 cm) eluted with 1% SDS/50 mM-Tris/2 mM-$MgCl_2$ buffer, pH 7.8. SDS/PAGE showed no contamination with non-digested G-actin or the *C*-terminal segment (227–375 sequence).

Immunological techniques

Polyclonal antibodies specifically directed against the 95–113 actin sequence were obtained from rabbit antisera induced by the 40–113 actin fragment and fractionated as described by Méjean *et al.* (1988). The 1–7 and 18–28 antibodies were described by Roustan *et al.* (1986).

Antibodies directed against S-1 were raised in rat according to Robin *et al.* (1976) and induced with the 27 kDa *N*-terminal segment of rabbit S-1 tryptic derivative prepared by the method of Mornet *et al.* (1980).

E.l.i.s.a. was used to monitor the interaction between coated actin and the antibody population in the presence or absence of S-1, and the interaction between S-1 and coated monomeric actin or actin peptides (Méjean *et al.*, 1987).

F-actin (200 μg/ml), G-actin (0.6 μg/ml), 1–226 *N*-terminal actin fragment (5 μg/ml) and 40–113 actin fragment (50 μg/ml) were coated on the microtitre plates and incubated overnight at 4 °C. Various concentrations of antibodies were incubated in the presence of various concentrations of S-1 (0–200 μg/ml). Each assay was made in triplicate. The mean of the error was less than 10%.

Western blotting was carried out as previously described (Roustan *et al.*, 1982).

SDS/PAGE

Electrophoresis was carried out on 5–18% (w/v) gradient polyacrylamide (Laemmli, 1970) and on 12.5% (w/v) polyacrylamide slab gels. Peptide bands were located with Coomassie Blue.

ATPase assay

The Mg^{2+}/actin-activated ATPase activities of S-1 were measured as described elsewhere (Mornet *et al.*, 1979) in a medium (1 ml) containing 10 mM-KCl/2.5 mM-ATP/1.5 mM-

$MgCl_2$ and 50 mM-Tris/HCl buffer, pH 8.0. The actin concentration varied from 2.5 to 25 μM. S-1 and S-1–actino-peptide-1 linked complex were added at 0.5 μM.

The Mg^{2+}-ATPase activities of S-1 or S-1–actino-peptide-1 were measured in the same buffer, and the protein concentration was 10 μM.

RESULTS

Involvement of the *N*-terminal actin sequence (1–28) in the S-1 interaction

The rigor acto–S-1 complex was treated with the hydrophilic zero-length cross-linker EDC, isolated, depolymerized, and digested as described in the Materials and methods section.

Analysis of thrombic digest by gel electrophoresis showed a typical protein pattern with the major typical band at 100 kDa (Bertrand *et al.*, 1988), as illustrated in lanes a and b of Fig. 1(*a*). We observed a minor band which could correspond to a 1–40 actino-peptide or a different conformational state of the actino-peptide-1–S-1. The actin segment cross-linked to the intact 95 kDa heavy chain of S-1 was identified by immunoblotting using 18–28 antibodies with the 1–28 actin segment (Fig. 1*a*, lane c). Cross-linking occurred on acidic residues 1–4 (Sutoh, 1982; Elzinga, 1987), but the 1–7 antibodies are unable to detect cross-linked products (Fig. 1*a*, lane d).

This covalently conjugated S-1 retained its ability to combine with fresh F-actin and was totally dissociated from actin with magnesium pyrophosphate (results not shown).

When the S-1–actino-peptide-1-complex-containing digest solution was used in ATPase kinetic analysis, concentrations of the complex were estimated as described in the Materials and methods section.

The S-1–actino-peptide-1 covalent complex solution, which is completely devoid of free S-1 (Fig. 1*a*), totally lost its elevated Mg^{2+}-dependent ATPase. In the presence of various concentrations of F-actin (Fig. 1*b*), its $V_{max.}$ value was 2-fold lower than that of the control S-1, but its $K_{app.}$ was unaffected; the same results were obtained with the cross-linked S-1 present in the whole digest or after its isolation by co-sedimentation with F-actin. A preparation of un-cross-linked acto–S-1 was treated under the same conditions without a decrease in the $V_{max.}$ of S-1.

As the concentration of the 100 kDa covalent complex was identical with that of S-1, the lower value of $V_{max.}$ could be due to the presence of the actino-peptide-1 covalently linked to the myosin head by EDC; previous experiments using antibodies (Das Gupta & Reisler, 1989*a*,*b*) or peptides (Van Eyk & Hodges, 1989) which interfere with the 1–7 segment–S-1 interaction are in agreement with this interpretation. The eventual inhibition by thrombic-digest products (K, L and M) appears improbable, as the ratio of these peptides to F-actin is low.

These results suggest that the *N*-terminal part of the actin sequence positioned on S-1 after cross-linking and proteolysis is unable to affect S-1–actin binding.

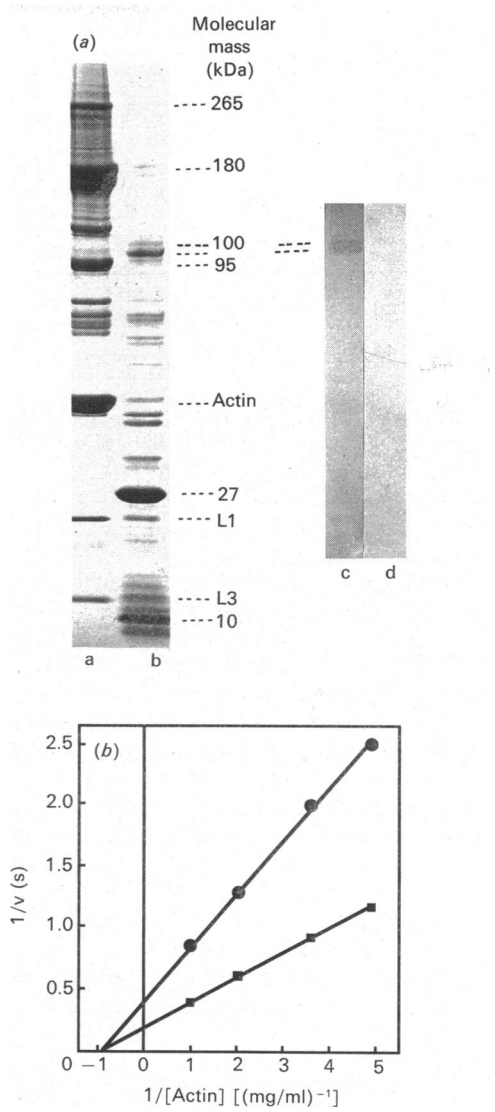

Fig. 1. Properties of actino-peptide-1–S-1 EDC complex

(*a*) Electrophoretic patterns on a 5–18% gradient gel of the tested EDC acto–S-1 before (lane a) and after (lane b) thrombic digestion and dissociation. The 27 kDa and 10 kDa bands correspond to L and M actin fragments respectively. The concentration of actino-peptide-1–S-1 was estimated by densitometric measurements of the 100 kDa band using S-1 heavy chain as standard. Lanes c and d show the antigenic reactivities of electrophoretic replicates from thrombic digest with 18–28 antibodies (lane c) and 1–7 antibodies (lane d). (*b*) Lineweaver–Burk plot of actin-activated ATPase of control S-1 (■) and thrombin-released EDC actino-peptide-1–S-1 complex (●). $K_{app.}$ was 25 μM, and $V_{max.}$ values were 5.0 s^{-1} and 2.3 s^{-1} for ■ and ● curves respectively. L1 and L3 are alkali light chains.

Direct interaction of the myosin head with monomeric actin and involvement of its 1–226 *N*-terminal sequence in the S-1 interaction

The participation of other sites outside the 1–28 *N*-terminal region is strongly suggested by the various approaches described above. In particular, the 40–113 segment has been shown to be in the vicinity of S-1 (Bertrand *et al.*, 1988). Thus we have been led to search these postulated sites in the *N*-terminal half of actin. Under our conditions S-1 showed an affinity of the same order for coated actin monomer, coated filamentous actin (not shown) and coated 1–226 actin derivative, as measured by e.l.i.s.a. (Fig. 2). Consequently, antibodies to the 40–113 actin segment (Méjean *et al.*, 1988; Benyamin *et al.*, 1988) were used as immunological probes to monitor possible binding of S-1 in this region. It has previously been shown (Méjean *et al.*, 1988) that the related epitopes located near residue 103 retain a similar accessibility to antibodies in unfolded, monomeric or polymeric actin. The apparent K_D for these antibodies is given in Table 1.

Involvement of the 40–113 segment in the S-1 interaction

The effect of the 95–113 antibodies on the S-1 interaction was

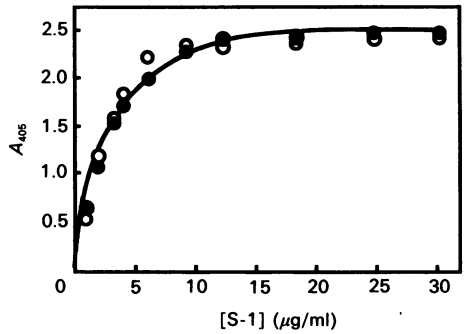

Fig. 2. Binding of S-1 to 1–226 *N*-terminal fragment

The 1–226 fragment (●) and G-actin (○) were coated in the same plate and S-1 was added at increasing concentrations (0–30 μg/ml) in 10 mM-Tris buffer, pH 7.8. S-1 was detected with anti-(27 kDa *N*-terminal S-1 fragment) antibodies by e.l.i.s.a.

Table 1. Estimation of dissociation constants of 95–113 antibodies and S-1 for actins and actin derivatives

Buffer A was 10 mM-Tris/HCl, pH 7.4. Abbreviation: nd, not determined.

	Apparent K_D			
	G-actin	F-actin	1–226 Actin fragment	40–113 Actin fragment
95–113 Antibodies (in buffer A)	20±5 nM	20±5 nM	nd	25±5 nM
S-1 (in buffer A)	20±5 nM	20±5 nM	20±5 nM	0.10±0.05 μM

then examined. As Fig. 3(*a*) shows, the binding of these antibodies was strongly affected by the presence of S-1 on monomeric actin. However, quantitative analysis showed that the maximum amount of antibodies able to react with monomeric actin was not S-1-dependent (Fig. 3*b*). The apparent K_D for 95–113 antibody varied and differed by a factor of about 10 (20–200 nM) in the absence of S-1 or at a saturating concentration of S-1 (Fig. 3*b* and 3*c*).

Similarly, S-1 binding appeared to differ by a factor of 10 (0.2×10^{-7} M–0.2×10^{-6} M) in the absence of antibody or at a saturating concentration (Fig. 3*d*). A similar effect was obtained with F-actin (results not shown).

These results are indicative for a strong, but partial, competition between 40–113 antibodies and S-1. Thus it was of interest to search for a possible interaction between the fragment involved (40–113 sequence) and S-1. The 40–113 peptide (50 μg/ml) purified over Ultrogel ACA-54 (Fig. 4) was coated, and e.l.i.s.a. results indicated that S-1 binds to the 40–113 fragment (Fig. 5) with an apparent dissociation constant of 1.0×10^{-7} M, which is higher than that for G-actin (Table 1). This difference in the S-1 affinity could be due to the peptide conformation being unsuitable at interaction. However, after 2

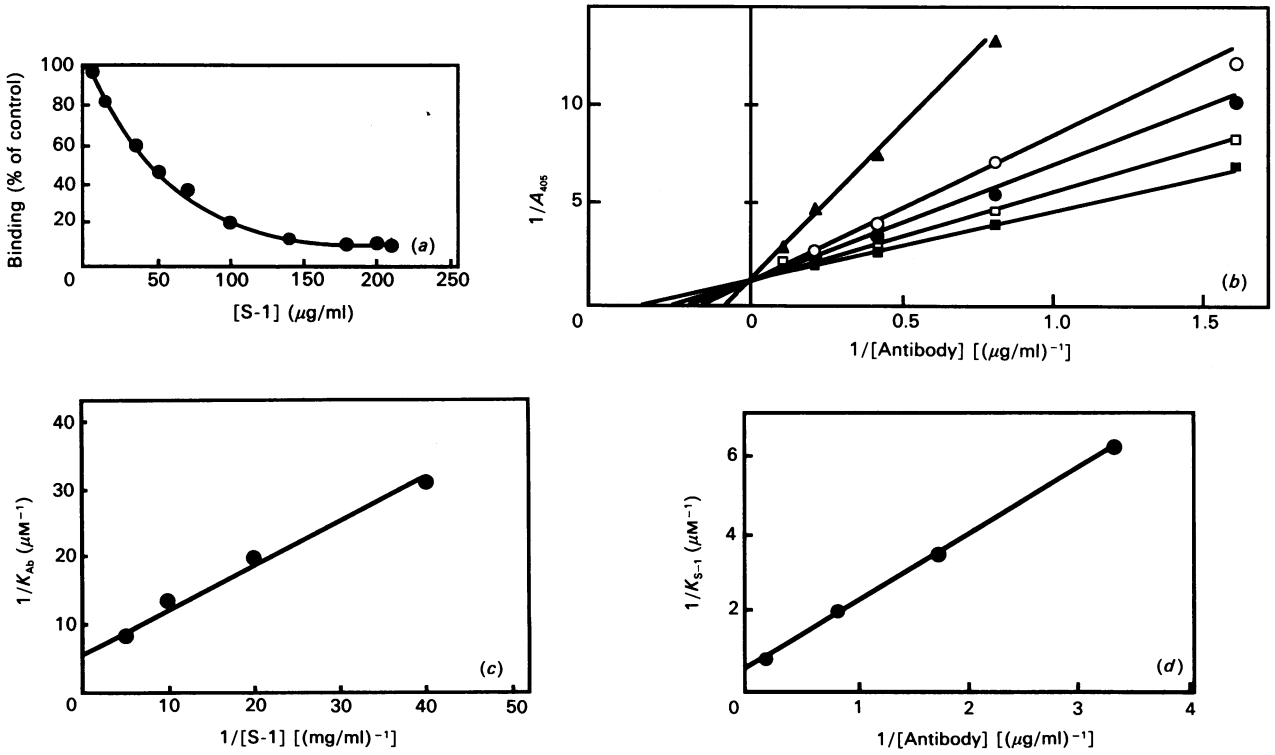

Fig. 3. Reactivity of 95–113 antibodies towards G-actin in the presence of S-1

The binding of specific anti-actin antibodies was monitored at 405 nm by direct e.l.i.s.a. as described in the Material and methods section. (*a*) Antibodies (0.6 μg/ml) were allowed to react with coated G-actin in the presence of increasing S-1 concentrations (0–250 μg/ml). (*b*) Antibodies at various concentrations (0.6–10 μg/ml) were allowed to react with coated G-actin in the presence of S-1 at several concentrations. A plot of $1/A_{405}$ versus 1/[Antibody] was drawn at the following concentrations: ■, 0 μg/ml; □, 25 μg/ml; ●, 50 μg/ml; ○, 100 μg/ml; ▲, 200 μg/ml. (*c*) Double-reciprocal plotting of apparent dissociation constants for antibodies (K_{app}) estimated from results in (*b*) versus S-1 concentrations. (*d*) Double-reciprocal plotting of apparent dissociation constants for S-1 versus antibody concentration.

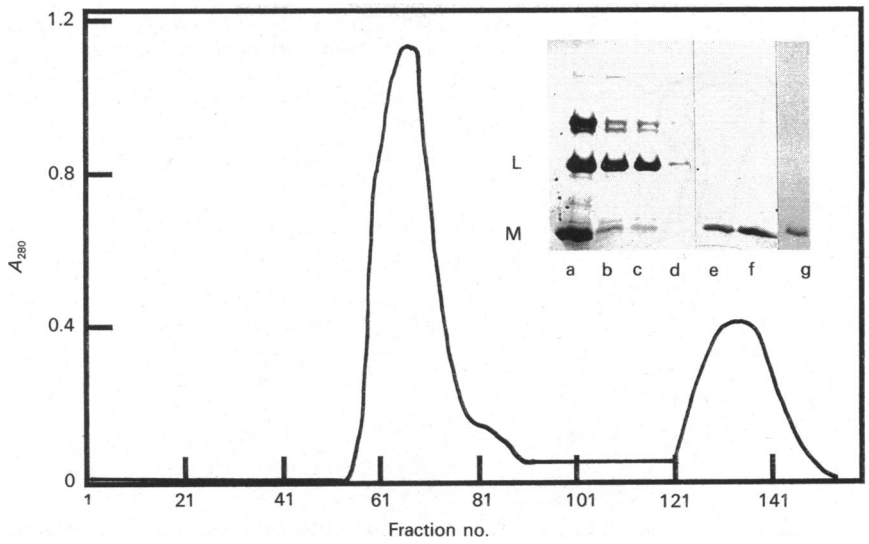

Fig. 4. Isolation of the 40–113 thrombic fragment of actin on Ultrogel ACA 54 column equilibrated with 10 mM-Tris/HCl buffer, pH 8

The absorbance of the fractions was measured at 280 nm. The flow rate was 25 ml/h and 2.0 ml fractions were collected. Inset: the SDS/polyacrylamide gels represent thrombic digests of actin (lane a) and fragments after elution (lanes b–f) (fractions nos. 69, 71, 134 and 138 respectively). Lane g shows the antigenic reactivity after electrophoretic blotting of the 40–113 peptide eluted from the column as revealed with 95–113 antibodies.

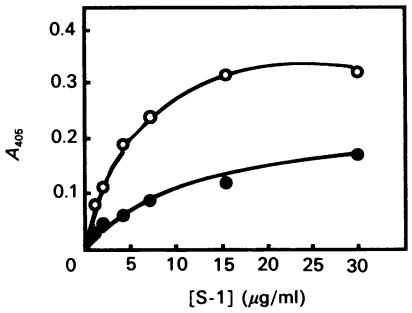

Fig. 5. Binding of S-1 to 40–113 fragment

The 40–113 fragment (●) and G-actin (○) were coated in the same plate and S-1 was added at increasing concentrations (0–30 μg/ml) in 10 mM-Tris buffer, pH 7.8. S-1 was detected by anti-(27 kDa *N*-terminal S-1 fragment) antibodies by e.l.i.s.a.

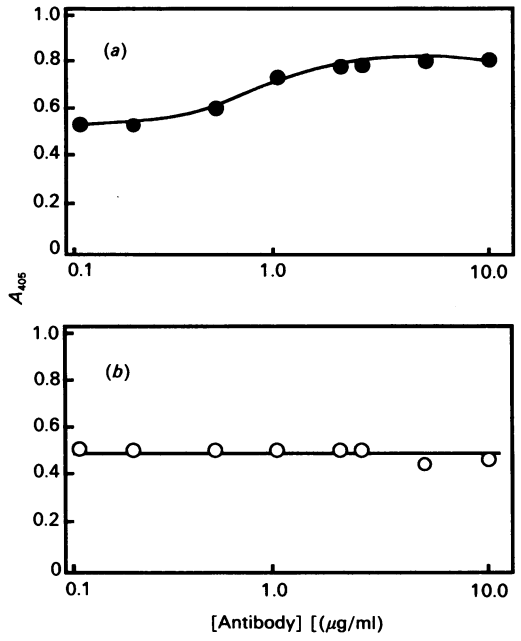

Fig. 6. Influence of 18–28 antibodies on 95–113 antibody binding

(*a*) 95–113 Antibody (10 μg/ml) interaction with coated G-actin (●) was monitored in the presence of increasing 18–28 antibody concentrations (0.1–10 μg/ml). (*b*) ○, 18–28 Antibody (3 μg/ml) interaction monitored in the presence of increasing 95–113 antibody concentrations (0.1–10 μg/ml).

or 3 days in solution, or after freezing, the preparation of 40–113 peptide showed conformational changes, which were detected by the loss of S-1 binding.

Long-range conformational relations between 18–28 and 95–113 sequences in the *N*-terminus of actin

We also observed that the 95–113 peptide, which could be taken as a structural entity on the basis of thrombic cleavage and its good retention of S-1 binding, appeared to have some relation with the *N*-terminal part of actin. As Figs. 6(*a*) and 6(*b*) show, there was an enhancement of 95–113 antibodies' reactivity on coated G-actin in the presence of increasing concentration of 18–28 antibodies. In contrast, no variation in 18–28 antibodies' reactivity was observed with increasing concentrations of 95–113 antibodies. These results, which are in good agreement with those from a previous study on the antigenic structure of actin (Benyamin *et al.*, 1988; Roustan *et al.*, 1988) provide evidence of the topological independence of the two epitopes and suggest that some interactions in the 18–28 region would be able to modify the exposition of residues in the 95–113 sequence.

DISCUSSION

Several aspects of the acto–myosin interaction are associated with information provided by the actin filament organization and its relationship with the monomeric structure and its primary sequence. Thus we discriminate between the co-operative process linked to the polymeric structure of actin, which could explain the mutual effect between S-1 and microfilaments, and their

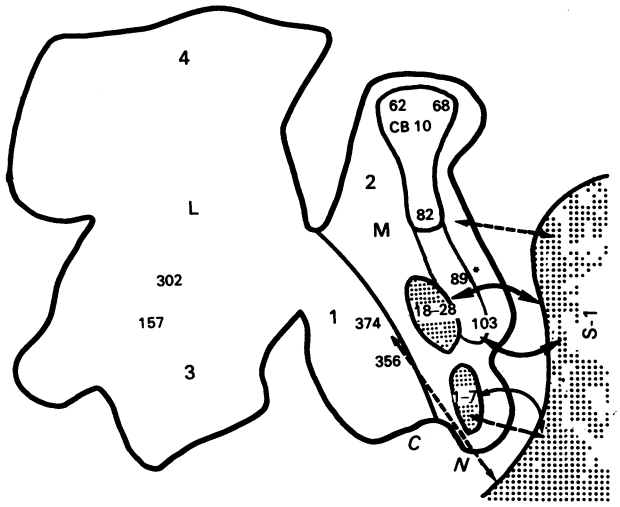

Fig. 7. Diagrammatic representation of rigor actin–S-1 interaction

⌒, Strong influence; ⌒ weak influence; ←---→ cross-linking. *N* and *C* are *N*- and *C*-termini. Values in small type are amino acid residue numbers. L and M are L and M actin fragments. The numbers in larger type (1, 2, 3 and 4) refer to subdomains of actin. CB 10 is the 48–82 peptide.

binding-site organization on the actin monomer, in which each interface would appear to allow information exchange between the myosin head and actin according to the ATPase kinetic steps.

A major difficulty in analysing the actin monomer results from its immediate polymerization under the critical concentration, which is induced by S-1, a myosin derivative that retains high solubility at low ionic strength. Immobilization of the actin monomer on Sepharose by covalent linkage (Chantler & Gratzer, 1973; Bottomley & Trayer, 1975) has shown that a specific interaction can occur between insoluble monomeric actin and myosin derivatives (heavy meromyosin or S-1), in which the complexes are reversibly dissociated at low ATP concentrations. Moreover, our previous studies have shown that monomeric actin coated on plastic plates at a subcritical concentration (0.6 μg/ml) can bind DNAase-1 or gelsolin (Boyer *et al.*, 1985, 1987; Benyamin *et al.*, 1988) and show conformational changes in the presence of cations (Méjean *et al.*, 1988), as reported in studies using actin in solution. Our present results provide evidence that coated monomeric actin is able to bind S-1 with a strong affinity under our experimental conditions (K_D = 0.020 μM) in the rigor state. The K_D value was of the same order of magnitude as that obtained by Chaussepied & Kasprzak (1989).

Such an approach could afford an easier way of exploring the 'multi-site interface model' postulated in the Introduction on the basis of previous results.

In the course of this investigation, we first analysed the covalent complex S-1–actin-(1–28)-peptide generated by EDC cross-linking of the rigor complex, depolymerization and thrombic proteolysis (Labbé *et al.*, 1986; Bertrand *et al.*, 1988). In contrast with the initial covalent complex, this entity is devoid of elevated Mg^{2+}-dependent ATPase activity, but retains its ability to combine with fresh actin and can be 're-activated'. In relation to the importance of the 1–28 region as a binding site (Méjean *et al.*, 1987) and its role in ATPase activity (Das Gupta & Reisler, 1989*a*,*b*) as well as its ability to retain these properties in a peptide configuration (Van Eyk & Hodges, 1989), our results suggest the existence of another region of actin for the S-1 interaction. These two regions could be carried by the same actin monomer or by two different monomers. The two binding sites have the same affinity.

We have previously suggested (Bertrand *et al.*, 1988) that there are contacts between S-1 and actin in the 40–113 actin region. We therefore used 1–226 and 40–113 fragments in the binding experiments. After coating on plastic plates, these peptides bound to S-1 with affinity constants equal to, or less than, 10^{-7} M. This result, which confirms the 'multi-site interface model' hypothesis can be refined. Thus spectral perturbations induced by the S-1–actin interaction and observed in ^{1}H n.m.r. experiments were quantitatively similar for the 1–44 and 1–82 actin fragments, which considerably localizes a binding region (Moir *et al.*, 1987) in the *N*-terminal sequence and not in the 45–82 sequence.

Moreover, the presence of an antigenic epitope near residue 103 carried by the 95–113 actin sequence led us to test for possible competition between 95–113 antibodies and S-1. We observed a strong, but partial, competition between S-1 and antibodies to coated monomeric actin which increased the apparent dissociation constant of S-1 and antibodies. These results indicate that the two sites are vicinal, but overlapping seems to be improbable. This interpretation is strengthened by the presence of two amino acid substitutions at positions 103 and 89 of the actin sequence. The former is observed in scallop muscle actin without any effect on S-1 binding (Hue *et al.*, 1988), in contrast with the latter, which may explain the different affinities of skeletal muscle S-1 to striated- and smooth-muscle actins (Strzelecka-Golaszewska & Sobieszek, 1981).

Thus two binding sites for S-1 on actin may be located: (i) in the 18–28 sequence (Méjean *et al.*, 1986, 1987) close to the *N*-terminal extremity, where a relationship to the ATPase activity of the myosin head has been shown (Das Gupta & Reisler, 1989*a*,*b*); and (ii) in the 40–113 segment, directly involving the 82–113 sequence.

The affinities of S-1 for coated monomeric actin and coated 1–226 peptide, which are of the same order, lead us to ask the following questions. Does binding of the S-1 occur separately at sequences 18–28 or 40–113 or at both? If both sites are involved, could the two forms exist in equilibrium? Could both sites react at the same time? We can also postulate that the binding site of S-1 is composed of two subsites which simultaneously react.

Structural relationships between these two regions cannot be excluded in the absence of high-resolution crystallographic analysis of actin. However, in a previous experiment (Benyamin *et al.*, 1988) it was shown that antibodies specific for epitopes including the 18–28 and 95–113 sequences are non-competitive for the actin monomer and that ternary complex can be obtained. However, some relation between the two regions seems probable, as shown by the present results (Fig. 6), which evidence some conformational relationships between them. Thus increasing concentrations of anti-18–28 antibodies raised the reactivity of anti-M antibodies for coated monomeric actin.

The possibility of a link between a conformational state of actin and the quality of interactions with the myosin head has been suggested by Konno's (1987) work and the preliminary results of Kwon *et al.* (1989). In the former, the 'split actin', an actin derivative cleaved at position 45, where the two fragments remain attached, retained its polymerization properties and a strong affinity for the S-1 in the rigor state, but showed a lower activating capacity. In the latter, intramolecular cross-linking in the actin monomer enhanced actin-activated Mg^{2+}-dependent ATPase without modifying other actin properties. Thus the hypothesis of information exchange between S-1-binding sites transferred via the actin monomeric structure is quite plausible.

In the present work, the spatial location of two S1-binding sites, which may be in close communication, was studied in the rigor state. These two regions (1–28 and 95–113) of actin sequence

considered in this work are in the same subdomain 2, and a myosin-binding site in this region (Fig. 7) is in agreement with the three-dimensional structure of G-actin (Kabsch & Holmes, 1990; Kabsch *et al.*, 1990). Subdomain 1 involves residue S^{14}, G^{15} and K^{18} and the *N*-terminal part 1–7.

It was demonstrated by Moir & Levine (1986) that two closely sequential segments of the primary structure of actin residues 62–74 (CB 10) and 83–90 (CB 11) as sites of contact with DNAase 1 are situated in the subdomain 2.

The polypeptide at the *C*-terminal end of subdomain 3 (peptide L) crosses over the cleft to subdomain 1, which also contains the *C*-terminus and involves the sequence residues 356–374. This last sequence was cross-linked with light chain by EDC (Sutoh, 1982).

In conclusion, the 'multi-site interface model' postulates that acto–S-1 interaction in the presence of nucleotide might be associated with particular conformations related to the ATPase nucleotide intermediates. These conformational changes might involve the *C*-terminal part of actin. Preliminary studies in this area have been described (Roustan *et al.*, 1988).

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