Antigenic Probes Locate the Myosin Subfragment 1 Interaction Site on the N-terminal Part of Actin

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The interaction of two different anti-actin antibody populations with the myosin subfragment 1-F-actin rigor complex has been studied. In contrast with the 1–7 sequence, the 18–28 sequence appears to be strongly implicated in the contact area of the myosin head on the actin polypeptide chain.

INTRODUCTION

We recently reported (1) the use of an immunological method as a valuable tool for describing the interface region of the actin molecule in the DNAse I-actin complex. This study showed that some parts of sequence 168–226 in actin are included in the contact area between these proteins.

In a parallel study, we have employed a similar approach to probe the involvement of the N-terminal part of actin in the rigor complex between F-actin and the myosin fragment S1. This stable step forms when no nucleotide is bound to S1 (2). The structure of the rigor complex is known mainly from three-dimensional image reconstruction of electron micrographs, which show the orientation of the S1 fragment toward the µ filament axis (3). However, other methods are needed to localize these interactions at the amino acid sequence level. Several approaches, i.e. chemical cross-linking, amino acid substitutions in actin isoforms, H1N.M.R., etc., have been widely used. It has been reported (4, 5) that there is a covalent cross-link between the 20 K and 50 K fragments of S1 and the N-terminal acidic amino acids of actin (4). The cross-link
Furthermore, Mossakowsk and Strzeleckagolaszewska (1985) (7) reported that different behaviors of myosin are correlated with substitutions of Cys by Val at position 17 in actin, and is consistent with hydrophobic as well as ionic interactions.

Finally, the involvement of the N-terminal region (1–44) are also supported by H1 N.M.R. studies (8).

The aim of this paper was to delimit precise regions in the N-terminal actin sequence (1–39) concerned with $S_1$ binding. Specifically, it appears that the site of myosin interactions includes the 18–28 actin sequence.

MATERIALS AND METHODS

Rabbit skeletal muscle actin was obtained according to the method in reference (9). Actin was proteolyzed by $S. aureus$ $\alpha$ protease (10) and trypsin (11). The small N-terminal tryptic peptides (related to the 1–68 residues of actin) were separated from the actin core as previously described (12). S-Carboxymethylated actin and N-terminal tryptic peptides were coupled to Sepharose 4 B as previously described (12). Large actin fragments derived from $S. aureus$ $\alpha$ protease cleavage were purified on a Sephacryl $S_{300}$ column (250 x 3 cm) equilibrated with 1 mM DDT, 1 mM EDTA, 0.5% SDS and 0.05 M Tris buffer, at pH 8.0.

Myosin was isolated from rabbit skeletal muscle according to the method described by Offer et al. (13). $S_1$ was prepared by digestion of myosin with chymotrypsin (14). Antibodies directed toward $S_1$ were elicited in rats using $S_1$ heavy chain unfolded in 1% SDS as the immunogen. Antibodies to rabbit F-actin were obtained as described by Benyamin et al. (6). Anti I gG antibodies labeled with peroxidase or alkaline phosphatase were from Byosis (France). Two antibody populations directed toward the N-terminal parts of actin (1–28) were obtained by affinity chromatography on insolubilized S-carboxymethylated actin and small N-terminal tryptic peptides, as previously described (12).

Protein concentrations were determined spectrophotometrically (15, 16, 17). Amino acid analysis and automatic sequence determination were performed as previously described (18–19). Protein and peptides were transferred to nitrocellulose as described by Roustan et al. (20).

The enzyme-linked immunosorbent assay (ELISA) technique was used to monitor the interaction between actin or $S_1$ and the antibody population as previously described (18), except that instead of 2% BSA, we used 0.5% gelatin + 3% gelatin hydrolysate, in the case of actin, and 5% protamin sulfate, in the case of $S_1$, to saturate all free protein binding sites.

RESULTS

Purification and Identification of the 19000 Fragment

In a previous paper (6) we showed that antisera induced by $F$-actin contain...
antibody populations specific for the 1–39 sequence of actin. We reported that two epitopes are located in this region (12).

To substantiate the location of the first epitope (Fig. 1) we studied the interaction of the corresponding antibody population toward an N-terminal 19000 fragment derived from *S. aureus* V8 protease cleavage of actin. This large fragment results (10) from a secondary cleavage site at Glu 167 of actin. In the present work, we isolated this fragment (see Methods) on a Sephacryl S300 column in the presence of SDS. The pooled fractions displayed a major component on slab gel (Fig. 2), with a molecular weight of about 19000. This fragment was submitted to an Edman degradation, and the major component (~80%) had the following sequence: Thr-Thr-Ala-Leu-Val-Cys-Asp-AsN-Gly. Two minor components (~5 and ~15%) had sequences of Gly-Tyr-Ala-Leu-Pro-His-Ala-Ile-Met and Met-Ala-Thr-Ala-Ala-Ser, respectively. The first sequence can be unambiguously identified as the 5–13 sequence of actin, and the two others can be related to the 168–176 and 227–232 sequences, respectively (Fig. 1).

Amino acid analysis of the fragment, as shown in Table 1, agrees with the calculated composition of sequence 5–167 in skeletal muscle actin, which confirms position 167 as a cleavage site for the protease. After electrophoretic transfer of a V8 hydrolysate of actin, the 19000 fragment did not respond to the specific population related to the first epitope (Fig. 2).

**Interaction of S1 with Insolubilized F-actin**

The interaction of S1 with F-actin coated on microtitration plates was tested using antibodies specific for the myosin subfragment. Figure 3 shows the results obtained. By
Fig. 2. Antigenic reactivity of the 19,000 fragment.
A. SDS slab gel electrophoretic identification of the purified fragment: (a) *S. aureus* V₈ protease digest of actin after 5 min hydrolysis. Molecular weight standards: thrombic digest of actin (23). (b) Purified fragment.
B. Antigenic reactivity of the fragment towards antibody specific for the N-terminal extremity of actin: (c) SDS slab gel electrophoresis of a *S. aureus* V₈ digest of actin after 12 min hydrolysis supplemented with actin. (d) Electrophoretic replicate.

Table 1. Amino acid composition of the 5–167 fragment derived from *S. aureus* V₈ protease digestion of actin. The calculated values are based on the known actin sequence.

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Total residues calculated</th>
<th>Proportion (mol/100 mol) calculated</th>
<th>Proportion (mol/100 mol) found</th>
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<tr>
<td>Asp</td>
<td>16</td>
<td>10.67</td>
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<td>Thr</td>
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<td>7.32</td>
</tr>
<tr>
<td>Ser</td>
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<td>4.67</td>
<td>3.03</td>
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<tr>
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<td>6.67</td>
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</tr>
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<td>Tyr</td>
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directly analyzing the binding curves as well as replotting these data, we estimated the $K_D$ of $S_1$ for insolubilized F-actin ($K_D = 0.45 \pm 0.05 \mu M$). This data is in reasonable agreement with results obtained by others (for example, a $K_D$ of 0.33 $\mu M$ is reported in reference 21) at a similar ionic strength and in homogeneous phase.

**Implication of the N-terminal Part of Actin in the $S_1$ Interaction**

The N-terminal part of actin (1–39) can be mapped by the two populations (related to the 1–7 and 18–28 sequences) purified from anti F-actin antibodies after affinity chromatography (see Methods, and ref. 12). The effect of each antibody population on the $S_1$- F-actin interaction was tested by the ELISA technique (see Methods). As shown in Figure 4, the two kinds of purified antibody exhibited different reactivities with the $S_1$-actin complex. The interaction of the first antibody population (specific for 1–7) was slightly affected by the presence of $S_1$ on F-actin ($S_1$ was used, under our experimental conditions, at a saturating concentration ($S_1 \sim 10K_D$). In contrast, the binding of the second antibody population (specific for the 18–28 sequence) to the $S_1$-F-actin complex was almost completely abolished.

**DISCUSSION**

The interaction of the myosin head with the actin filament in the rigor complex involved both the 50 K and 20 K fragments of $S_1$ and the light chain $A_1$ (4, 5).

Several studies (4, 6, 7, 8) have shown that $S_1$ is involved in the interaction with the
N-terminal extremity of actin (sequence 1–44 or smaller) while a part of the light chain A1 (1–41 sequence) (22) interacts with the C-terminal region of actin (4, 8). Thus, the contact area must include the two actin extremities, which are in spatial proximity as shown previously (1).

Based on our results, and in agreement with Levine and Moir (8), the N-part of actin (sequence 1–44) is located near or at the S1 interaction site. This conclusion is drawn from the following observations. The binding of anti actin antibodies (specific for the N-terminal sequence) is affected by S1 binding. The S1 binding site can be studied more precisely using 2 distinct epitopes located in the 1–28 sequence.

Population 1 is specific for a very limited portion of the actin sequence. Our data show that the 19000 peptide beginning at position 5 in the sequence is not recognized by population 1, which indicates that the 1–4 sequence is included in the first epitope (Fig. 1). This was already demonstrated by the fact that the antibodies discriminated between skeletal and cardiac muscle actin, which can only be distinguished by an inversion at positions 2 and 3 (12) Fig. 1). This antibody population 1 is weakly affected by S1 interaction. Thus, region 1–7 seems to be at least near, but not directly in contact.
with the $S_1$ site. Moreover, this hypervariable sequence in actin seems to be related to a modulation of $S_1$ activity. For instance, in the case of smooth muscle, substitution of Cys instead of Val at position 17 in the actin polypeptide chain enhances the $K_{app}$ for myosin ATPase activation. This effect can be explained by the induction of a conformational change in this region and probably by long-range structural variations (7).

In contrast, the constant 18–28 region related to antibody population 2 (12, Fig. 1) must be directly related to $S_1$ binding, since the formation of a ternary $S_1$-antibody-actin complex does not appear to be possible.

In conclusion, the $S_1$ actin contact area includes the 18–28 sequence of actin, probably in connection with the C-terminal extremity. The involvement of the last region is now being studied.

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REFERENCES