Structural and functional variations in skeletal-muscle and scallop muscle actins

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Structural and functional properties in two striated-muscle actins, one from a vertebrate, the other from an invertebrate (scallop), were compared in relation to a smooth-muscle actin isoform (aortic actin). In spite of differences in the variable *N*-terminal region, the two striated-muscle isoactins showed, in contrast with aortic actin, a large structural homology revealed by proteinase-susceptibility and interaction with the myosin head. Thus the myosin head may bind to the two striated-muscle actins in constant parts of the 18–113 sequence. In contrast, antigenic reactivity of conformational epitopes of these actins strongly differentiated scallop actin from the two others. The behaviour of the scallop actin appears to be related to several amino acid substitutions located near or at functional domains such as monomer–monomer binding site, DNAase-I-dependent actin–actin binding site and actin-severing domain, which modified the polypeptide chain exposure.

INTRODUCTION

Comparison of the amino acid sequence of actins from various sources shows that this molecule is highly conserved from an evolutionary viewpoint. For instance, rabbit skeletal-muscle actin is 95% homologous with actin from an invertebrate (scallop) (Vandekerckhove *et al.*, 1983). Furthermore, most of the substitutions are considered to be conservative amino acid replacements (Hightower & Meagher, 1986). This behaviour may reflect high selective pressure, resulting in the retention of protein folding related to actin–binding-protein interactions. In spite of this noticeable homology, actins exhibit some differences in their functional properties. For example, it has been reported that the Mg^{2+} -dependent ATPase activity of the skeletal-muscle myosin head differs depending on whether it is activated by striated-muscle or smooth-muscle actins (Cavadore *et al.*, 1985a; Strzelecka-Golaszewska & Sobieszek, 1981). Polymerization parameters also depend upon the actin isoform (Khaitlina, 1986).

The most variable part of actin is its acidic *N*-terminal sequence, on which vertebrate isoactin classification is based (Vandekerckhove & Weber, 1984). Three major types have been defined. Skeletal-muscle and cardiac striated-muscle actins are of the α -type, smooth-muscle actins of the α - and γ -types and cytoplasmic actins of the β - and γ -types (Bulinski *et al.*, 1983). Invertebrate actins, which have been less well analysed (De Couet, 1983), share homologous sequences with cytoplasmic actins (Vandekerckhove *et al.*, 1983). For instance, scallop adductor-muscle actin has been characterized as a β -like actin (Khaitlina, 1986), in contrast with its location in a striated muscle (Millman & Bennett, 1976).

The proposed involvement (De Couet, 1983; Hambly *et al.*, 1986) of this variable extremity (1–12 sequence) of actins in the reported functional variation has not been confirmed (Mossakowska & Strzelecka-Golaszewska, 1985; Strzelecka-Golaszewska *et al.*, 1985). This raises questions as to the location and the nature of the amino acid substitutions along the primary structure and the related consequences for the biological role of the isoactin expressed in a particular tissue.

In the present study we compared structural, immunological and biological properties of rabbit skeletal-muscle and scallop adductor-muscle actins with a non-striated-muscle actin (aortic actin). The observed differences are discussed in relation to the location of substitutions along the sequence.

MATERIALS AND METHODS

Proteins

Bovine aortic actin was prepared as described elsewhere (Cavadore *et al.*, 1985a). Actins from skeletal muscle (rabbit) and scallop striated adductor muscle (*Pecten maximus*) were obtained from acetone-dried powders (Spudich & Watt, 1971). Filamentous actins were labelled at Cys-374 with 1,5-I-AEDANS by the procedure of Takashi (1979). Rabbit skeletal-muscle and scallop muscle myosins were obtained as described by Offer *et al.* (1973) and Stafford *et al.* (1979) respectively. S1 was prepared by digestion of myosin with chymotrypsin and purification by gel filtration on Sephacryl S200 (Stafford *et al.*, 1979; Weeds & Taylor, 1975).

Anti-(sheep IgG) or anti-(rabbit IgG) antibodies conjugated with alkaline phosphatase or peroxidase were purchased from Biosys (Compiègne, France).

Abbreviations used: 1,5-I-AEDANS, *N*-(iodoacetyl)-*N'*-(5-sulpho-1-naphthyl)ethylenediamine; S1, subfragment 1 of myosin.

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Protein concentrations

Protein concentrations were determined spectrophotometrically by using the following absorption coefficients: actin, $A_{290}^{0.1\%} = 0.637$; S1, $A_{280}^{0.1\%} = 0.75$; IgG, $A_{280}^{0.1\%} = 1.50$.

Polyacrylamide-slab-gel electrophoresis

Polyacrylamide (12.5% or 15%)-slab-gel electrophoreses were carried out in the presence of 0.1% SDS as described by Laemmli (1970). Gels were scanned with a Shimadzu C.R. 3A densitometer.

Proteolytic cleavages of actin

Monomeric actins in 2 mM-Tris/HCl buffer, pH 7.6, containing 0.2 mM-ATP and 0.1 mM-CaCl₂ or filamentous actins labelled with 1,5-I-AEDANS in 50 mM-Tris/HCl buffer, pH 7.6, containing 0.1 M-KCl, 0.1 mM-ATP and 2 mM-MgCl₂ were digested by trypsin (enzyme/actin ratio 1:10, w/w) at 20 °C. Monomeric actin in the presence of 1 mM-EDTA was subjected to thrombin cleavage as described by Muszbek & Laki (1974). *Staphylococcus aureus*-V8-proteinase cleavages were performed in the presence of 1% SDS and with a proteinase/actin ratio of 1:50 (w/w) as previously described (Roustan *et al.*, 1985). In all cases samples were withdrawn at the appropriate times and analysed by SDS/polyacrylamide-slab-gel electrophoresis.

Immunological techniques

Antibodies to rabbit skeletal-muscle and bovine aortic smooth-muscle filamentous actins were induced as previously described (Benyamin *et al.*, 1986; Cavadore *et al.*, 1987) with the use of glutaraldehyde-stabilized filamentous actins. These antibodies display a strong reactivity to sequence 1–28 (Benyamin *et al.*, 1986; Cavadore *et al.*, 1987). Anti-(scallop muscle actin) antibodies were also raised in rabbits with the use of glutaraldehyde-stabilized filamentous actin as the antigen. The immunization procedure is described in detail elsewhere (Benyamin *et al.*, 1986). In all cases antibody populations directed against discontinuous epitopes were purified by affinity chromatography (Cavadore *et al.*, 1985b). These purified populations exhibited a negative reaction to unfolded actin, as determined by immunoblots (results not shown) and Roustan *et al.* (1986). The reactivity of the antibody populations towards filamentous actin was determined by direct enzyme-linked immunosorbent assay as previously described in detail (Méjean *et al.*, 1987).

ATPase assay

Actin-activated S1 Mg²⁺-dependent ATPase activity was determined at 25 °C for 20 min in the presence of 50 mM-Tris/HCl buffer, pH 8.0, containing 10 mM-KCl, 5 mM-ATP and 5 mM-MgCl₂. The concentration of filamentous actin was increased in increments from 1.25 μM to 40 μM, and S1 was added at 1 μM. Phosphate release was determined by using an automated analysis described by Mornet *et al.* (1979).

Electron microscopy

Glycerinated scallop adductor muscle (Wells & Bagshaw, 1983) was fixed with 2% (v/v) glutaraldehyde in 0.1 M-sodium cacodylate buffer, pH 7.2. It was then washed with 0.16 M-cacodylate and post-fixed with 1% OsO₄ for 60 min at 4 °C, and subsequently dehydrated

and embedded in Epon. Thin sections, contrasted with uranyl acetate and lead citrate, were examined with a JEOL 2000 EX electron microscope. The lengths of thin and thick filaments were measured on 11 individual filaments and at several magnifications, and standard deviations were determined.

Location of hydrophobic regions in the polypeptide chain

This was done by the method of Hopp & Woods (1981), in which the average hydrophilicity of hexapeptides 1–6, 2–7... along the amino acid sequence was calculated. Calculations were performed on a Macintosh microcomputer.

RESULTS

Proteolytic cleavages of isoactins

Tryptic digestion. Comparative proteolysis of monomeric actin from skeletal muscle and scallop muscle, performed under the same experimental conditions, were used previously to compare skeletal-muscle and aortic smooth-muscle actins (Cavadore *et al.*, 1985a). The two striated-muscle actins gave the same pattern in SDS/polyacrylamide-gel electrophoresis after tryptic cleavage (Fig. 1), characterized by a major band at M_r 33000, which was also observed (Cavadore *et al.*, 1985a) in the case of smooth-muscle actin. The cleaved positions have been located (Jacobson & Rosenbusch, 1976) at residues 61/68 in a constant part of the actin sequence (Table 1).

The cleavage of scallop muscle and skeletal-muscle filamentous actins was also studied (Fig. 1) after labelling Cys-374 with a fluorescent reagent. After proteolysis the electrophoretic mobility of these actins remained unchanged, but a total loss of the fluorescent label was observed. This confirms the limited cleavage of filamentous actin at position 372 (De Couet, 1983). Moreover, the rate of fluorescence release was the same for both actins (Table 2), whereas it differs between smooth-muscle and skeletal-muscle filamentous actins (Cavadore *et al.*, 1985b).

Thus it can be concluded that scallop and rabbit striated-muscle actins, as well as smooth-muscle actin, show a similar susceptibility to trypsin proteolysis at positions 61/68 and 373, but the environment of the C-terminal extremity in smooth-muscle actin appears to differ from that of the striated-muscle actins.

Thrombin and *S. aureus*-V8-proteinase proteolysis. The two actins were proteolysed by thrombin in the presence of EDTA as described by Muszbek & Laki (1974). As shown in Fig. 1 and Table 2, the results obtained do not differ significantly with respect to either the size of the fragments produced, as evaluated by SDS/polyacrylamide-gel electrophoresis, or the time course of cleavage.

Similar results were obtained with *S. aureus* V8 proteinase. As in the case of skeletal-muscle actin (Roustan *et al.*, 1985), scallop actin was not cleaved by this proteinase in the absence of SDS (Fig. 1). The time course of actin fragment release was therefore studied in the presence of 1% SDS. The yield of the various fragments obtained from the two actins was compared. The yield of the 19000- M_r fragment, which is derived from the N-terminal 26000- M_r fragment (Roustan *et al.*, 1985), was low in both cases (Fig. 2). By contrast, the

Fig. 1. Comparative cleavage patterns of skeletal-muscle (Sk) and scallop muscle (Sc) actins by various proteolytic enzymes

Polyacrylamide slab gels are stained by Coomassie Blue in (a), (c) and (d) and irradiated with u.v. light in (b). The electrophoretograms show proteolysis by trypsin of monomeric (a) and 1,5-I-AEDANS-labelled filamentous (b) actins, and by thrombin (c) and by *S. aureus* V8 proteinase (d) of monomeric actins.

Table 1. Amino acid mutations (in the 7–113 sequence) for various actins

Actin source	Residue					
	10	16	17	76	89	103
Skeletal muscle*	Cys	Leu	Val	Ile	Thr	Thr
Vascular smooth muscle*	Cys	Leu	Cys	Ile	Ser	Thr
Non-muscle β -type†	Val	Met	Cys	Val	Thr	Val
Non-muscle γ -type†	Ile	Met	Cys	Val	Thr	Val
Scallop adductor muscle*	Val	Met	Cys	Val	Thr	Val

* Vandekerckhove *et al.* (1983).
† Vandekerckhove & Weber (1984).

26000- M_r fragment from skeletal-muscle actin was more stable than that from scallop actin. In the latter case the fragment was easily degraded into a smaller product of M_r about 25000 (Fig. 2). However, the half-time of the degradation of the two actins was the same (Table 2). In contrast, previous studies have demonstrated a difference in the susceptibility of aortic smooth-muscle

Table 2. Rate of digestion of actin by proteinases

Half-times ($t_{1/2}$) are given as ranges of values for three determinations.

Proteinase	$t_{1/2}$ (min)	
	Skeletal-muscle actin	Scallop muscle actin
Trypsin*	2.0–2.5	2.0–2.5
Thrombin†	40–60	40–60
<i>S. aureus</i> V8 proteinase†	0.8–1	0.8–1

* On filamentous actin, digestion is monitored by fluorophore release.

† On monomeric actin, digestion is monitored after Coomassie Blue staining.

actin to thrombin (Cavadore *et al.*, 1985a) and to *S. aureus* V8 proteinase (Cavadore *et al.*, 1985b), which is cleaved at the same positions, i.e. 113 (Muszbeck & Laki, 1974) and 226 (Cavadore *et al.*, 1985b), but at different

Fig. 2. Time course of the cleavage of scallop muscle (a) and skeletal-muscle (b) actins by *S. aureus* V8 proteinase

Digests were subjected to SDS/polyacrylamide-gel electrophoresis, and the gels were stained with Coomassie Blue and scanned at 590 nm. The amounts of 26000- M_r (▲), 25000- M_r (●) and 16000- M_r (■) fragments (arbitrary units) are plotted versus hydrolysis time.

rates, although these two cleavage sites are both located in constant parts of actin sequences (Vandekerckhove *et al.*, 1983).

Activation of S1 Mg²⁺-dependent ATPase activity by isoactins

The activity of S1 purified from rabbit skeletal muscle and scallop adductor muscle was monitored in the presence of increasing concentrations of filamentous actin (see the Materials and methods section). The maximum activation of S1 ATPase by F-actins was found to be closely related to the actin origin (Fig. 3). ATPase activity (V_{max}) was higher in homologous systems than in heterologous ones, but the apparent K_m was identical in homologous and heterologous systems (Fig. 3).

In contrast, it has been reported (Prochniewicz & Strzelecka-Golaszewska, 1980; Strzelecka-Golaszewska & Sobieszek, 1981) that vertebrate smooth-muscle and striated-muscle actins show different behaviours. Skeletal-muscle myosin (or heavy meromyosin) was activated (V_{max}) to the same extent by striated-muscle (skeletal or cardiac) and smooth-muscle (aortic or gizzard) muscle actins, but the apparent K_m values were different.

Fig. 3. Activation of skeletal-muscle (a) and scallop muscle (b) myosin-head Mg²⁺-dependent ATPase by skeletal-muscle (●) and scallop muscle (■) actins

The reciprocal of the enzymic activity is plotted versus the reciprocal of the actin concentration.

Comparison of the antigenic reactivity of isoactins

The cross-reactivity of scallop muscle, aortic smooth-muscle and skeletal-muscle filamentous actins was tested by direct enzyme-linked immunosorbent assay (Méjean *et al.*, 1987). Purified antibody populations directed against discontinuous epitopes were selected (see the Materials and methods section) to compare the conformations of the filamentous actins. As shown in Fig. 4, aortic smooth-muscle and skeletal-muscle actins showed similar antigenic reactivities when tested against the three antibody populations. In contrast, scallop muscle filamentous actin was strongly differentiated by homologous and heterologous antibodies. These results reveal that the higher mutation content, which differentiates scallop muscle actin from the other two actins (Vandekerckhove *et al.*, 1983), has a strong effect on the antigenic activity of the exposed structures of scallop muscle filamentous actin.

Thin-filament length in scallop adductor-muscle myofibrils

Electron-microscopic analysis of scallop adductor-muscle myofibrils revealed typical striated-muscle patterns [lengths of the sarcomere (2.5 μm), thin filaments (1.19 ± 0.09 nm) and thick filaments (1.88 ± 0.15 nm) as previously reported (Huddart, 1975; Millman & Bennett, 1976). Nevertheless, the length of the actin filaments, measured from the M band extremity to the middle of

Fig. 4. Immunoreactivity of isoactins monitored by enzyme-linked immunosorbent assay

Scallop muscle filamentous actin (▲), aortic smooth-muscle filamentous actin (●) and skeletal-muscle filamentous actin (■) were coated on micro-titre plates. Actins were treated with (a) anti-(skeletal-muscle actin) antibodies, (b) anti-(aortic smooth-muscle actin) antibodies and (c) anti-(scallop muscle actin) antibodies.

Fig. 5. Effect of amino acid exchange on the average hydrophilicity values in variable regions in the skeletal-muscle actin sequence

(a) Location of some functional domains and mutations along the amino acid sequence. $\longleftrightarrow$, DNAase-I-dependent actin-actin binding site; $\blacktriangleleft***\blacktriangleright$, actin-severing domain. The number of amino acid exchanges is calculated for each 30-amino-acid-residue interval. We have reported comparison between skeletal-muscle and aortic smooth-muscle actins (■), between skeletal-muscle and scallop muscle actins (□) and between aortic smooth-muscle and scallop muscle actins (▨). (b) Hydrophilicity patterns of skeletal-muscle actin (—) and scallop muscle actin (---).

the Z disc, was slightly greater (1.19 ± 0.09 nm) than reported by Millman & Bennett (1976) (1.01 ± 0.09 nm) (results not shown).

DISCUSSION

Although sequences and basal functions of all actin isoforms are strongly conserved, several lines of evidence substantiate the concept that some of the observed amino acid substitutions are not conservative replacements but are directly related to functional differences between striated-muscle, smooth-muscle and cytoplasmic actins. Our present results extend to an invertebrate muscle actin the concept that striated-muscle actins display tissue-specificity in their conformation as well as in some of their functional properties, although scallop muscle actin possesses a sequence largely related to cytoplasmic actins.

Skeletal-muscle and scallop muscle actins showed a very similar susceptibility to proteolysis by trypsin, thrombin or *S. aureus* V8 proteinase at or near positions 39, 62, 113, 167, 226 and 373. A comparative study previously demonstrated (Cavadore *et al.*, 1985b) significant differences in the case of smooth-muscle actin. Since the cleaved positions are located in conserved sequences of the three actins, it can be concluded that the local arrangement of these sequences is indistinguishable in skeletal-muscle and scallop muscle actins and somewhat different in aortic smooth-muscle actin.

Moreover, in the presence of ATP the affinities (apparent K_m values) of rabbit skeletal-muscle and scallop adductor-muscle actins for skeletal-muscle S1 were identical. Under comparable conditions, previous studies have shown similar apparent K_m values for skeletal-muscle and cytoplasmic actins (Gordon *et al.*, 1977), but different values (by a factor greater than 2.5-fold) in the case of smooth-muscle actins (Mossakowska & Strzelecka-Golaszewska, 1985). Recent studies (Méjean *et al.*, 1987; Bertrand *et al.*, 1988) allow one to restrict the binding contacts (in the rigor state) of actin on the S1 heavy chain to discrete parts of sequence 18–113. Moreover, the peptide CB13 (sequence 1–44) and the thrombic fragment (40–113) retain the ability to interact with S1 (Moir *et al.*, 1987; Labbé *et al.*, 1988). Thus it is tempting to correlate the variations in the apparent affinity with substitutions occurring in the sequence 18–113. From the data reported in Table 1 it can be seen that only three amino acid substitutions differentiate actins in this region. No evident correlation appears between substitutions at positions 76 and 103 and the reported apparent K_m of actins for S1. In contrast, the substitution at position 89 apparently correlates with differences observed in S1 associations. Therefore the substitution at position 89 would exert a significant effect on the interaction between actin and myosin.

Immunological analysis of filamentous actin revealed large conformational changes (Fig. 4) between skeletal-muscle and scallop muscle actins, in contrast with the limited variations observed between skeletal-muscle and aortic smooth-muscle actins. A direct relationship between the number of amino acid replacements and the immunological cross-reactivity is evident when comparing (Fig. 5a) the number of replacement sites for aortic smooth-muscle actin (eight) and scallop muscle actin (21) with skeletal-muscle actin as the reference. It can be observed (Fig. 5a) that the replacement sites

specific for scallop muscle actin are located in the central, and the C-terminal, parts of the sequence. The antibodies related to the hypervariable N-terminal region were eliminated by affinity chromatography and are not considered here.

With regard to the conservative or non-conservative nature of these replacements, four of them (Fig. 5b) appear to be able to modify the hydrophilicity of the polypeptide chain and, thereby, its exposure.

Three of the replacements (Val-103, Ala-129 and Thr-201) are located on hydrophobic portions of scallop muscle actin at or near the functional domain described as the DNAase-I-dependent actin-actin binding site (Boyer *et al.*, 1985; Hambly *et al.*, 1986). The last replacement site (Ser-365) is located in hydrophilic portion of the C-terminal part and directly concerns a short sequence (Asp-Glu-Ser-Gly) proposed as part of the actin-severing domain (Ampe & Vandekerckhove, 1987; Kwiatkowski *et al.*, 1986) near the second actin-actin binding site, which is profilin-dependent. The location of these four replacement sites near the actin-actin binding domains raises questions as to the polymerization properties and filament stability of scallop muscle actin. In spite of its higher critical concentration for polymerization and its greater susceptibility to DNAase-I depolymerization and to shearing forces (Khaitlina, 1986) (compared with skeletal-muscle actin), which appear to be related to the location of the four substitution sites, we observe that these properties *in vitro* are not reflected by the length of the μ filament in the scallop muscle sarcomere. This length is somewhat longer than in the case of the skeletal-muscle myofibril (Huddart, 1975). A possible explanation could be a stabilization effect exerted *in situ* by tropomyosin, which, in the absence of troponin-like proteins (Millman & Bennett, 1976), is deprived of the Ca^{2+} regulation provided by the myosin molecule in scallop adductor muscle.

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