Structural variations in actins

Biochemical and immunological tools for probing the structure of rabbit skeletal-muscle and bovine aortic actins

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Structural differences between skeletal-muscle and aortic actins were studied by using biochemical and immunological approaches. By using proteinase digestion we found that three regions of actin show structural differences: (a) in the C-terminal part, (b) the region around residue 227 and (c) the region around residue 167. By using antibodies specific to particular actin conformations we can discriminate between monomeric and filamentous forms of the two actins. Our results show that the minor sequence variations of the N- and C-terminal regions induce structural change in these regions, but also some long-range structural variations in other regions.

Actin, which is one of the major elements of the contractile system in muscles, is known to be a highly conserved protein (Vandekerckhove & Weber, 1979). In mammals, none of the known primary sequences of skeletal muscle actins show any variation (Eggerston, 1968; Gallucci & Garen, 1966). In mammalian smooth muscles, the amount of each isoactin expressed depends on the muscle origin (Vandekerckhove & Weber, 1981; Small & Sobieszek, 1983). Moreover, rabbit skeletal-muscle actin is more than 97% homologous with bovine aortic actin (Bulinski *et al.*, 1982). In spite of their homology, these two actins exhibit different functions *in vitro*. The variable extent of Mg^{2+} -ATPase activation in skeletal-muscle myosin, caused by skeletal- or smooth-muscle actins (Strzelecka-Golaszewska & Sobieszek, 1981; Cavadore *et al.*, 1985) shows that the actin–myosin interaction depends on the nature of the actin. The actin-activated Mg^{2+} -ATPase of myosin can be modulated by tropomyosin. It has been shown that there is no direct competition between actin and tropomyosin for the same binding site on myosin (Sobieszek, 1982). The modulation of the actin-activated Mg^{2+} -ATPase of myosin results from the specific interaction of actin and tropomyosin and was shown to depend on the nature of both actin and tropomyosin (Nosaka *et al.*, 1984; Williams *et al.*, 1984; Yamaguchi *et al.*, 1984).

In the present paper we report the use of biochemical and immunological approaches to evidence structural differences between the actins. The proteolytic susceptibility of the two actins was used to demonstrate different accessibilities to the cleavage sites located in the central part and in the C-terminal region. The C-terminal sequence has been shown to be close to the N-terminal region (Sutoh & Mabuchi, 1983; Boyer *et al.*, 1985) where most of the sequence changes are concentrated. Antisera induced by filamentous skeletal-muscle actin, cleared from antibodies directed against the variable N-terminal

part, were also used in another independent approach to determine structural variations between these closely related actins.

MATERIALS AND METHODS

Skeletal-muscle and aortic actins were prepared as described by Pardee & Spudich (1982) and Cavadore *et al.* (1985) respectively. Actin was labelled on cysteine 374 by 1,5-I-AEDANS according to Takashi (1979). *Staphylococcus aureus* V_8 proteinase was from Miles laboratories, and trypsin was from Sigma. Goat anti-rabbit antibodies conjugated with alkaline phosphatase or peroxidase were purchased from Biosys, Compiègne, France. CNBr-activated Sepharose was from Pharmacia; actins were immobilized on this support.

Protein concentrations

These were determined by the method of Spector (1978), with γ -globulin as the standard. Alternatively, protein concentrations were determined by using the following absorbances: actin $A_{290nm}^{0.1\%} = 0.63$; IgG, $A_{280nm}^{0.1\%} = 1.5$.

Polyacrylamide-gel electrophoresis

This was carried out in the presence of 0.1% SDS as described by Laemmli (1970), by using the discontinuous buffer system and 15% (w/v) polyacrylamide. Gels were scanned with a Shimadzu C-R 3A densitometer.

Amino acid and sequence analyses

These were performed on Beckman model 120 C analyser and a Socosi sequenator respectively. Automatic sequence determination was performed as described elsewhere (Jauregui-Adell *et al.*, 1982).

Abbreviations used: 1,5-I-AEDANS, N-iodoacetyl-N-1-naphthyl-5-sulphoethylenediamine; SDS, sodium dodecyl sulphate; phosphate-buffered saline, 140 mM-NaCl/10 mM-KH₂PO₄/10 mM-Na₂HPO₄, 2.6 mM-KCl, pH 7.4; e.l.i.s.a., enzyme-linked immunosorbent assay; Mg^{2+} -ATPase, Mg^{2+} -actin activated ATPase.

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Proteolytic cleavage of actin with immobilized or soluble *S. aureus* proteinase

Immobilization procedure. *S. aureus* proteinase (5 mg) dissolved in 0.1 M- Na_2CO_3 containing 0.5 M-NaCl was added to 10 ml of pre-swollen CNBr-activated Sepharose and gently rotated end-over-end for 3 h at room temperature. After washing and saturating the reacting sites with ethanolamine, the enzyme linked to Sepharose was stored at 4 °C.

A portion of the resin was dried and hydrolysed for 24 h with 6 M-HCl at 110 °C in order to determine the amount of enzyme linked to the resin. A 50–60% yield was generally obtained under these conditions.

Cleavage procedure. The immobilized *S. aureus* proteinase was added to a solution of actin containing 1% SDS (proteinase/actin ratio 1:50, w/w) and the mixture was rotated end-over-end at 25 °C. Samples were withdrawn at the appropriate time, after separation of the resin by rapid centrifugation, and subjected to 0.1% SDS/15%-polyacrylamide-gel electrophoresis. Proteolysis of actin by soluble proteinase was performed at the same enzyme/actin ratio and the same temperatures. The resulting digests were cleared of SDS by use of an Extracti-gel D column (Pierce, Rockford, IL, U.S.A.) and fractionated on a μ -Bondapak C_{18} column (Waters).

Tryptic cleavage of actins

F-actins labelled with 1,5-I-AEDANS were digested in 50 mM-Tris/HCl, (pH 7.8)/0.2 mM-ATP/0.1 mM- CaCl_2 by trypsin (enzyme/actin 1:10, w/w) at 20 °C. Under these conditions, trypsin removes a small peptide from F-actin that contains cysteine-374 (De Couet, 1983). Samples were withdrawn at the appropriate times and subjected to 0.1% SDS/15%-polyacrylamide-gel electrophoresis. Fluorescence was quantified by using a Shimadzu densitometer equipped with a fluorescence attachment, and the gels were stained with Coomassie Blue.

Immunological techniques

Antibodies against rabbit skeletal-muscle filamentous actin were induced by using trinitrophenylated F-actin (Benyamin *et al.*, 1983). These antibodies display a limited reactivity against the 1–62 sequence (Roustan *et al.*, 1985) and a major reactivity (70% of the antibodies elicited) against the F-conformation of actin. The antibodies specific for the conformational epitopes were selected and cleared of the activity against sequential epitopes by immunoadsorption to an unfolded-carboxymethylated-actin-Sepharose column. This column was obtained by immobilization of skeletal actin after carboxymethylation in 8 M-urea, on Sepharose CL6B by using the CNBr coupling procedure. The non-adsorbed antibodies exhibited a negative reaction against unfolded actin on immunoblot. Peptide transfer from SDS/polyacrylamide-gel electrophoresis to nitrocellulose sheets (0.2 μm -thick) was performed as previously described (Roustan *et al.*, 1982).

E.l.i.s.a.

F- or G-actins were diluted to 200 $\mu\text{g}/\text{ml}$ in 0.5 M- $\text{Na}_2\text{CO}_3/\text{NaHCO}_3$ buffer (pH 9.6)/0.01% NaN_3 . A 150 μl portion was added to the wells of 96-well U-bottom microtitre plates. After overnight incubation at 4 °C, the

Fig. 1. SDS/15% polyacrylamide gel electrophoresis of the cleavage products obtained by cleavage with *S. aureus* V_8 proteinase

(a) Skeletal-muscle actin digest; (b) aortic actin digest; (c) 1,5-I-AEDANS-labelled aortic actin digest. Lanes (a) and (b) were stained with Coomassie Blue; lane (c) shows bands visible under u.v. light.

antigen was removed and the plates were automatically washed (Titertek microplate washer 120; Flow Laboratories) with phosphate-buffered saline containing 0.01% NaN_3 and 0.5% Tween 20.

The plates were incubated with 2% (w/v) bovine serum albumin for 2 h at room temperature in order to saturate all free protein-binding sites. After washing with phosphate-buffered saline/Tween buffer, plates were again incubated for 2 h at room temperature with 2% bovine serum albumin. Dilutions of the immune serum were added to the wells and incubated for 12 h at 4 °C, after which the wells were washed five times with phosphate-buffered saline containing 150 mM-KCl. Goat anti-rabbit IgG conjugated with alkaline phosphatase (1:1000 dilution) was added and incubated for 2 h at room temperature. After washing, a solution of *p*-nitrophenyl phosphate (1 mg/ml) was added to the wells and incubated 30 min at room temperature. A_{405} was read automatically in a Titertek multiscan instrument (Flow Laboratories).

RESULTS

Proteolytic cleavage of actins

Comparative proteolyses of skeletal-muscle and aortic actins with *S. aureus* V_8 proteinase were performed as described in the Materials and methods section.

SDS/polyacrylamide-slab-gel electrophoresis showed the presence of three major bands of M_r 30000, 21000, 16000 and some minor faint bands, both for skeletal-muscle and aortic actins (Fig. 1). The two actins labelled with 1,5-I-AEDANS on cysteine-374 were cleaved with *S. aureus* proteinase. The digests were subjected to gel

Fig. 2. Time course of the cleavage of aortic actin (a) and skeletal-muscle actin (b) by soluble (i) or immobilized (ii) *S. aureus* V₈ proteinase

The experimental conditions were as described in the Materials and methods section. The digests were subjected to SDS/15% polyacrylamide-gel electrophoresis, stained with Coomassie Blue and scanned at 590 nm. The quantity of each fragment is expressed as a percentage of the total versus time. ●, actin; ○, 30000 M_r ; ■, 21000 M_r ; ▲, 16000 M_r .

Fig. 3. Kinetic analysis of the proteolysis of skeletal-muscle (□, ■) and aortic (○, ●) actin by soluble (□, ○) or immobilized (■, ●) *S. aureus* V₈ proteinase

The experimental conditions are identical with those of Fig. 2. A_0 and A represent the quantity of actin at zero time and at a given time.

electrophoresis. Fig. 1 shows that only the 16000- M_r peptide and the undigested actin were fluorescent, indicating that this peptide contains the C-terminal part of actin.

The time courses of actin cleavage for aortic and skeletal-muscle actins, are shown in Figs. 2(a) and (b) respectively. The yield of the various fragments depended on the nature of the actin (Fig. 2). The yield of the

Fig. 4. H.p.l.c. fractionation of the *S. aureus*-proteinase-digested fragments of aortic actin (a) and skeletal muscle actin (b)

Actins (1 mg) were digested as indicated in the Materials and methods section and injected into a 7.8 mm $\times$ 300 mm μ -Bondapak C₁₈ column. Elution was carried out at 3 ml/min for 40 min by using a 0–60% acetonitrile gradient in 0.01% trifluoroacetic acid. The eluate was simultaneously monitored at 280 nm (----) and 230 nm (—). The arrow indicates the position of the 16000 M_r fragment.

21000- M_r peptide was very large in the case of aortic actin and very small in the case of skeletal-muscle actin. By contrast, the 30000- M_r peptide rapidly degraded when aortic actin was used, but was very stable in the case of skeletal-muscle actin. The half-time ($t_{1/2}$) of the two actins was determined by kinetic analysis of the proteolyses (Fig. 3). Comparison of the $t_{1/2}$ values when soluble or immobilized proteinase was used shows substantial differences between the two actins. The $t_{1/2}$ values corresponding to skeletal-muscle actin and aortic actin were found to be 48 and 168 s for soluble proteinase, 144 and 396 s with immobilized proteinase.

Purification and identification of the actin fragments

The 16000- M_r fragment was purified by h.p.l.c. from both skeletal-muscle and aortic actins (Fig. 4). The two fragments were eluted at the same concentration of acetonitrile. Amino acid analysis of the two peptides did not indicate any significant difference (Table 1).

The skeletal-muscle and aortic 16000- M_r fragments were subjected to an Edman degradation, and both peptides had the following identical sequence:

Met-Ala-Thr-Ala-Ala-Ser-Ser-Ser-Ser-Leu

which was unambiguously identified as the N-terminal part of the sequence of the residues 227–375 region.

Similar results were obtained when immobilized proteinase was used instead of soluble proteinase. In this case, the secondary cleavages were minimized. The 21000- M_r peptide obtained from aortic actin was partially purified by h.p.l.c. (ascending part of the last peak in Fig. 4). After electrophoretic transfer to

Table 1. Comparative amino acid composition of the 16000- M_r peptides obtained by digestion of actins with *S. aureus* V₈ proteinase

Amino acid	Composition (residues/molecule)			
	Aortic actin		Skeletal-muscle actin	
	Found	Calculated*	Found	Calculated†
Asx	9.50	10	9.6	10
Thr	9.3	10	10	12
Ser	13.2	16	12.6	15
Glx	16.3	16	16	16
Gly	10.5	10	10.1	10
Ala	11.8	10	11.5	11
Val	4.4	5	4.2	4
Met	5.9	6	5.9	7
Ile	16.8	16	16.1	15
Leu	8.5	8	7.4	7
Tyr	5.3	6	5.6	6
Phe	5.2	5	5.3	5
His	1.9	2	1.9	2
Lys	10.1	9	9.4	9
Arg	5.9	6	6.7	6

* Calculated from sequence 227–375 in aortic actin.

† Calculated from sequence 227–375 in skeletal-muscle actin.

Fig. 5. Identification of the *N*-terminal fragment in the *S. aureus*-V₈-proteinase digest of aortic actin

(a) SDS/15% polyacrylamide gel, Coomassie Blue-stained; (b) electrophoretic replicate reaction with anti-(F-actin) serum. The standard markers are albumin (M_r 43000), carbonic anhydrase (30000), trypsin inhibitor (20100) and lactalbumin (14400).

nitrocellulose, the 21000- M_r peptide displayed a positive response with a specific anti-actin serum directed against the *N*-terminal sequence of actin (residues 1–62) (Fig. 5). This result indicates that the 21000- M_r peptide resulting from the degradation of the *N*-terminal 1–226-peptide of aortic actin includes the *N*-terminal part. Identical conclusions were already obtained by two (C.R. and Y.B.) of us in the case of skeletal-muscle actin (Roustan *et al.*, 1985).

Tryptic cleavage of 1,5-I-AEDANS-labelled F-actins

Cleavage of the labelled actins resulted in a loss of the fluorogenic part of the two actins (Fig. 6), but the

Fig. 6. Time course of the fluorescence decrease in 1,5-I-AEDANS-labelled skeletal-muscle actin (●) and aortic actin (▲) obtained by tryptic proteolysis

Coomassie Blue-stained gels did not reveal any apparent modification of the electrophoretic mobility or of the absorbance of the actin bands after proteolysis. However, the fluorescence release differed significantly for the two actins. This result suggests that the aortic actin has been cleaved at the same site as has the skeletal actin, but that the accessibility of this site is different in the two actins.

Immunological reactivity of skeletal-muscle and aortic filamentous actins

The cross-reactivity of skeletal-muscle and aortic filamentous actins was tested by the e.i.s.a. technique (Fig. 7). The two proteins exhibited different reactivities towards antibodies specific for conformational epitopes of filamentous skeletal-muscle actin. Moreover, skeletal-muscle and aortic actins lose their reactivity against the conformational antibodies when the actins are depolymerized before coating.

Fig. 7. Immunoreactivity of skeletal-muscle and aortic G- and F-actins revealed by e.l.i.s.a.

●, Skeletal-muscle F-actin; ○, aortic F-actin; ■, skeletal-muscle G-actin; □, aortic G-actin.

DISCUSSION

The present paper describes the use of immunological and biochemical approaches to probe structural differences between actins.

The biochemical approach shows that the two major cleavage sites indicated by digestion with *S. aureus* V₈ proteinase were found to be identical in both aortic and in skeletal-muscle actins. The amino acid compositions of the fragments of apparent M_r 16000 are in good agreement with those calculated from primary sequences (Vandekerckhove & Weber, 1979). The *N*-terminal sequences determined for the two fragments were identified as belonging to the beginning of the *C*-terminal segment, residues 227–375. On the basis of the above results we conclude that the main cleavage occurs both in aortic and skeletal-muscle actins at residue 227. Thus the resulting fragments have M_r values of 25390 and 16440 respectively, as calculated from the sequence data.

Our results show that a secondary cleavage site is present in actins. *S. aureus* proteinase is known to cleave preferentially at glutamic acid residues (Drapeau, 1976), and the secondary cleavage site was tentatively located at glutamic acid-167. The fragment with an apparent M_r of 21000 (M_r 18400 on the basis of amino acid composition) resulting from this secondary cleavage was derived from the 1–226 segment of actin. Since the cleavage products obtained with *S. aureus* proteinase were identical, the comparative proteolytic time courses of the two actins reveal significant structural changes or a possible differential dodecyl sulphate binding for the two actins. In all cases environments near to, or distant from, the major cleavage site are not similar.

The modification of the environment is more evident at the second cleavage site (residue 167). The rapid increase in the amount of the 21000-apparent- M_r fragment in the case of aortic actin (Fig. 2) was not evident with skeletal-muscle actin. The susceptibility of this peptide bond was also found to be very significant in *Dictyostelium* actin, which is related to cytoplasmic actins (Simpson *et al.*, 1984). Since there were no sequence variations near the cleavage sites, it seems reasonable to suppose that the two three-dimensional arrangements of the constant sequence are somehow different.

These structural variations, which concern the monomeric form of actin, are also observed with the filamentous form, as shown by the proteolytic and immunological approach. The marked difference between the two rates of tryptic hydrolysis is again an argument in favour of a structural variation between the two actins, but in this case the structural variation affects the *C*-terminal conformation in the filamentous form. These structural differences between the filamentous forms were confirmed by the differential immunological reactivities of the two actins. Some of the specific antibody(ies) that recognize the filamentous form are able to discriminate structural changes between the two actins. The present work, in which two independent approaches were used, provides evidence that the two actins are structurally different, which was already suggested by their different functional properties.

By using proteinases we found that three regions of actin display differential structural changes: (a) the *C*-terminal part, (b) the region around residue 227, and (c) the region around residue 167. Finally, by using conformational antibodies we were able to discriminate between monomeric and filamentous forms of skeletal-muscle and aortic actins.

Our result show that these weak sequence variations induce significant structural changes in the *N*- and *C*-terminal regions of actin and also some long-range structural changes in other region(s) of the molecule, which may explain the differential biochemical and immunological reactivities of skeletal-muscle and smooth-muscle actins.

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Received 19 April 1985/3 June 1985; accepted 18 June 1985