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The importance of C-terminal amino acid residues of actin to the inhibition of actomyosin ATPase activity by caldesmon and troponin I

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Proteolytic elimination of three C-terminal amino acid residues from actin weakens its interaction with caldesmon and troponin I and, in consequence, lowers the inhibitory effects of both proteins on actomyosin ATPase activity. These results prove the importance of C-terminal extremity of actin to the overall interaction of this protein with caldesmon and troponin I.

Caldesmon; Troponin; C-Terminus of actin; Actomyosin ATPase

1. INTRODUCTION

Smooth muscle, caldesmon, and striated muscle, troponin I, are the tissue-specific inhibitory components of thin filament regulatory systems of muscle contraction (for review see [1–4]). Association of each with actin is accompanied by the inhibition of actin-activated ATPase activity of myosin and suppression of contraction.

Several lines of experimental evidence from proton nuclear magnetic resonance spectroscopy, cross-linking and immunochemical studies have indicated that the binding sites with caldesmon and troponin I are located in the N-terminal region of actin [5–9]. Our recent investigations revealed, however, that IAEDANS label attached at C-terminal Cys-374 of actin in skeletal muscle ghost fibers was sensitive to the conformational changes occuring in actin structure upon binding of caldesmon or troponin I [10]. This observation together with the close proximity of C- and N-terminus of actin [11] suggested that the C-terminal amino acid residues may take part in the constitution of the recognition surface for these two proteins. The results presented here, showing that removal by trypsin of the three C-terminal amino acid residues of actin reduces the inhibitory effect of caldesmon and troponin on actomyosin ATPase, confirm this view.

2. MATERIALS AND METHODS

Caldesmon was isolated from chicken gizzards according to the method of Bretscher [12]. Rabbit skeletal muscle myosin was prepared according to Perry [13] and further purified following the procedure of Kielley and Bradley [14]. Rabbit skeletal muscle actin and troponin, as well as chicken gizzard tropomyosin, were prepared according to procedures described elsewhere [15].

Actin devoid of three C-terminal amino acid residues was obtained by digestion of Mg-ATP-G-actin with trypsin according to Strzelecka-Gołaszewska, H. and Mossakowska, M. (personal communication). The digestion of actin labelled with IAEDANS at Cys-374 (1 mg/ml) was carried out in buffer containing 0.1 mM $MgCl_2$, 0.2 mM ATP, 0.2 mM EGTA, 1 mM DTT, 0.02% NaN_3 and 2 mM HEPES, pH 7.6, at 1:50 (w/w) enzyme to substrate ratio for 60 min at room temperature. The completion of the reaction was monitored by the disappearance of the fluorescence of the actin band on SDS-gels. The reaction was terminated by adding SBTI (2:1 SBTI to trypsin, w/w). Digested actin was polymerized with 100 mM KCl and ultracentrifuged for 3 h at 100,000 × *g*. Sedimented pellet was homogenized in and dialysed against buffer containing 0.1 mM $CaCl_2$, 0.2 mM ATP, 1 mM DTT, 0.02% NaN_3 and 2 mM HEPES, pH 7.6, and again polymerized with 100 mM KCl.

The purity of all proteins was checked by SDS-PAGE on 7.5–20% gradient mini-slab gels according to Laemmli [16].

Protein concentration was determined by measuring UV light absorbance with the following values of extinction coefficients and molecular weights: G-actin, $E^{1\%}_{290nm} = 6.3$, 42 kDa [17]; myosin, $E^{1\%}_{279nm} = 5.4$, 470 kDa [18]; chicken gizzard tropomyosin, $E^{1\%}_{277nm} = 1.9$, 68 kDa [19]; caldesmon, $E^{1\%}_{280nm} = 3.8$, 87 kDa [20]; troponin, $E^{1\%}_{278nm} = 4.5$, 69 kDa [21].

ATPase activity of rabbit skeletal muscle actomyosin (reconstituted from F-actin, in amounts given in the Figure legends, and 200 μg/ml of myosin) was assayed at 30°C in a medium containing 50 mM KCl, 2 mM $MgCl_2$, 2 mM ATP, 2 mM EGTA and 10 mM imidazole-HCl, pH 7.0. The concentrations of caldesmon and troponin are given in the Figure legends. Tropomyosin, when present, was used at a molar ratio to actin of 1:6. The amount of P_i liberated was measured by the method of Fiske and SubbaRow [22].

Sedimentation experiments were performed at room temperature in a buffer containing 50 mM KCl, 2 mM $MgCl_2$, 0.1 mM EGTA, 2 mM β-mercaptoethanol and 20 mM imidazole-HCl, pH 7.0. Samples prepared by mixing of either caldesmon or troponin with F-actin (in proportions indicated in the Figure legends) were incubated for 30 min with stirring and ultracentrifuged using Airfuge (Beckman) for 30 min. Resuspended pellets were subjected to SDS-PAGE. Gels were stained with Coomassie brillant blue G-250 and the amount of caldesmon or troponin I bound to actin in pellets was quantified by laser scanning densitometry.

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3. RESULTS

3.1. *Activation of myosin Mg^{2+}-ATPase activity by truncated and intact actin*

Comparison of the activation profiles of myosin Mg^{2+}-ATPase by intact actin and actin devoid of three C-terminal amino acid residues revealed that at a low actin to myosin ratio the former was less effective than latter (Fig. 1). However, at higher ratio of these two proteins, starting from about 0.375:1 (w/w), the level of the ATPase of actomyosin containing truncated and intact actin was the same. Therefore, to study the effect of C-terminal residues of actin on the functioning of regulatory proteins, caldesmon and troponin, the actomyosin reconstituted from 80 μg/ml of actin of 200 μg/ml of myosin (i.e. at a 0.4:1 w/w ratio) was used.

3.2. *The effect of caldesmon on myosin Mg^{2+}-ATPase activated by truncated and intact actin*

Fig. 2 shows the effect of caldesmon on the ATPase of skeletal muscle actomyosin reconstituted from intact actin and actin devoid of C-terminal extremities. Titration of the ATPase with caldesmon both in the presence and absence of tropomyosin showed gradual inhibition of enzymatic activity, although in the case of truncated actin the inhibitory effect was reduced. This results from the lower affinity of caldesmon to truncated rather than intact actin (Fig. 2A, inset) and suggests that C-terminal residues take part in the binding of caldesmon to actin and their removal weakens the binding of both proteins. In control experiments it was shown that stimulation of actomyocin ATPase by tropomyosin and the tropomyosin–actin interaction are not affected by removal of C- terminal residues of actin (data not shown).

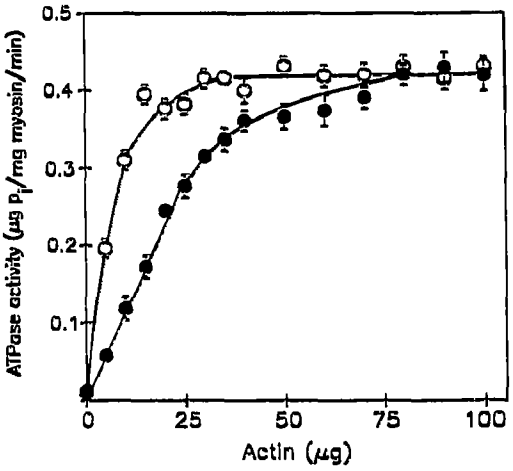

Fig. 1. Comparison of the activating effects of actin devoid of three C-terminal amino acid residues (●) and intact actin (○) on Mg^{2+}-ATPase activity of myosin. The ATPase activity was assayed in the medium containing 50 mM KCl, 2 mM $MgCl_2$, 2 mM ATP, 2 mM EGTA and 10 mM imidazole-HCl, pH 7.0, at 200 μg/ml of rabbit skeletal muscle myosin and actin concentrations indicated on the abscissa.

3.3. *The effect of troponin on myosin Mg^{2+}-ATPase activated by truncated and intact actin*

The influence of troponin on the ATPase activity of actomyosin containing either actin devoid of three C-terminal amino acid residues or intact actin was studied in the absence of Ca^{2+} and in the presence of tropomyosin. Under these conditions, the inhibition of actomyosin ATPase induced by troponin I, like that by caldesmon, was lower in the case of truncated actin (Fig. 3). These results correspond well with sedimenta-

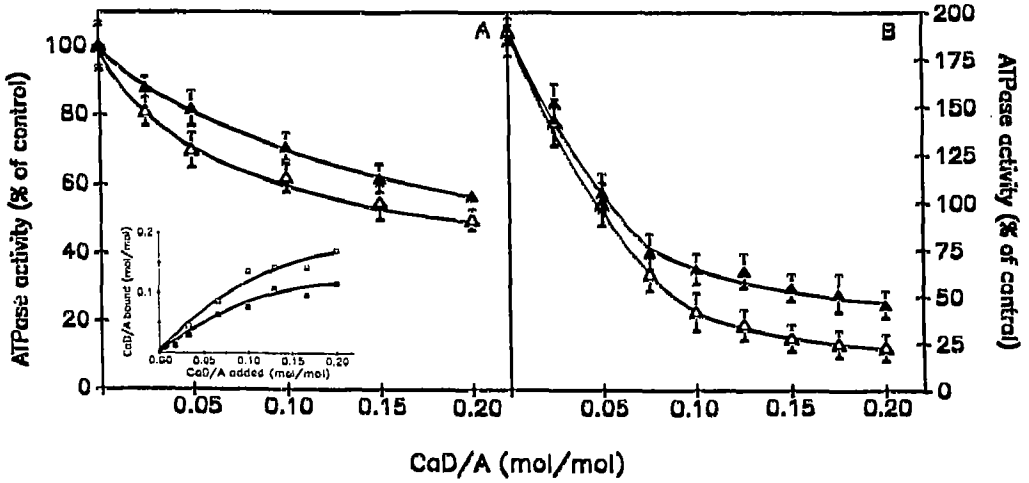

Fig. 2. The effect of caldesmon on the ATPase activity of actomyosin containing actin devoid of tree C-terminal amino acid residues (▲) and intact actin (△). The ATPase activity was assayed in the absence (A) and presence (B) of tropomyosin (at 1:6, molar ratio to actin) at actin and myosin concentrations of 80 and 200 μg/ml, respectively. Other conditions are as described in Fig. 1. The inset shows binding of caldesmon to truncated (■) and intact (□) actin. The samples of caldesmon were ultracentrifuged with actin. The pellets were subjected to PAGE and the molar ratios of caldesmon to actin were determined as described in Materials and Methods. CaD, caldesmon; A, actin.

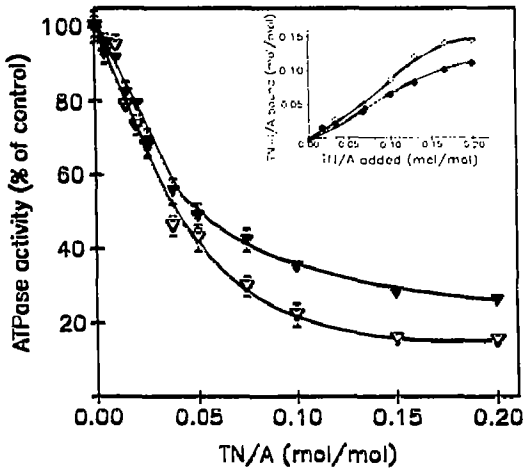

Fig. 3. The effect of troponin–tropomyosin complex on the ATPase activity of actomyosin containing actin devoid of three C-terminal amino acid residues (▼) and intact actin (▽). A molar ratio of tropomyosin to troponin of 1:1 was used; other conditions were as in Fig. 2. The inset shows binding of troponin I to truncated (●) and intact (◇) actin. The samples of troponin–tropomyosin complex (1:1, molar ratio) were ultracentrifuged with actin. The pellets were subjected to page and the molar ratios of troponin-I to actin were quantified as described in Materials and Methods. TN, troponin; TN-1, troponin I; A, actin.

tion data showing reduced binding of troponin I to truncated actin as compared with intact actin (Fig. 3, inset).

4. DISCUSSION

The interaction of F-actin with myosin heads is correlated with the large increase in actin-activated ATPase activity of myosin and muscle contraction. As a result of extensive studies it was established that, at various stages of ATP hydrolysis, three sequences of actin monomer are involved in the interaction with myosin subfragment 1. Two of them are situated in the N-terminal third of actin [23–27], while the third, responsible for the interaction with skeletal muscle myosin light chain 1, is located in the C-terminal region [28]. Removal of three amino acid residues from the C-terminus by tryptic digestion of actin decreased its binding affinity to myosin as reflected by the fact that at low actin to myosin ratios truncated actin was less efficient in the activation of myosin Mg^{2+}-ATPase activity than intact actin. The inhibitory effect of caldesmon and troponin on the ATPase activity of actomyosin reconstituted from truncated actin and myosin was lower when compared with that on intact actomyosin complex.

The tissue-specific inhibitors, caldesmon and troponin I, show a similar general mode of interaction with actin [10,29]. Each has two binding sites at the N-terminal region of actin and the binding to these sites is essential for their inhibitory effect on actomyosin ATPase activity [5,6]. According to the results presented in this paper the maximum inhibitory effect of both requires the integrity of the C-terminal portion of the actin polypeptide chain. Despite these similarities, due to the occupation by caldesmon and troponin I of non-identical but sequential sites within the N-terminal region of actin [5,6], the detailed inhibitory mechanism of actomyosin ATPase is different. Whereas caldesmon weakens the interaction of actin with myosin [1,30] as a result of the competitive displacement of myosin heads from the same or partially overlapping binding site(s) on actin [5], troponin I seems to be involved in the slowing down of the kinetic steps of ATP hydrolysis (P_i release) by the troponin–tropomyosin complex that takes place after the actin–myosin association [1,5,29,31].

The importance of C-terminal amino acid residues to the inhibition of actomyosin ATPase by caldesmon and troponin I can be explained in two ways. Since the C-terminal extremity of actin is situated in close proximity to the N-terminal binding site(s) [11], the C-terminal amino acids are either included in the construction of a common binding site with N-terminal binding sequence(s), or, due to some conformational changes evoked in the latter, may modify its interaction with inhibitory proteins. A similar conclusion was drawn in a recent preliminary report of Crosbie et al. [32] who applied fluorescence (pyrene-iodoacetamide label attached to Cys-374) and co-sedimentation methods to study the interaction of caldesmon with the C-terminal region of actin. On the other hand, very recent results of Murad et al. [33] showing with the use of proton nuclear magnetic resonance technique that the association of myosin light chain 1 with the C-terminal region of actin is accompanied by the weakening of troponin I binding to actin, are also compatible with our data.

All these results, together with the detected restriction of the relative motions of the C-terminal segment of actin protomers in filament upon interaction with caldesmon and troponin I [10], can be taken as further evidence of indirect or direct association of these proteins with the C-terminal extremity of actin.

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