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The Substitution of Troponin in Bullfrog and Chicken Myofibrils by Rabbit Skeletal Troponin, Troponin-T and Troponin-T₁

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Abstract The substitution of troponin in bullfrog skeletal myofibrils by rabbit skeletal troponin, troponin T and troponin T₁ was examined by the densitometry of the SDS gel-electrophoretic patterns. The troponin in the myofibrils was replaced by the added rabbit skeletal troponin and troponin T (tropomyosin binding component of troponin) to almost the same extent. On the other hand, troponin T₁, the N-terminal side fragment of two chymotryptic fragments of troponin T replaced troponin in the myofibrils less effectively than either troponin or troponin T. Troponin T, also replaced troponin in the myofibrils from rabbit and chicken striated muscles, less effectively than troponin T. This suggested that, in addition to the N-terminal side troponin T₁ region, the C-terminal side region (troponin T₂) of rabbit skeletal troponin T was also involved in the exchange reaction of troponin in the bullfrog skeletal myofibrils.

Introduction

Troponin is a Ca²⁺-receptive protein of myofibrils in vertebrate striated muscles. This protein is a complex of three different subunits, troponin C, I and T, and is distributed at regular intervals along the entire length of the thin filament. All three troponin subunits are required for the Ca²⁺-regulation of the contraction⁸⁾.

We recently showed that troponin in bullfrog skeletal myofibrils could be exchanged by rabbit skeletal or bovine cardiac troponin complex at slightly acidic conditions¹⁰⁾.

In the present paper, we examined the effect of treatment with troponin T and troponin T₁ from the rabbit skeletal muscle on bullfrog skeletal myofibrils.

Experimental procedures

Preparation of myofibrils

The myofibrils from skeletal muscle of bullfrog, rabbit and chicken were prepared by the procedure of Harigaya et al., and stored in a 50% glycerol solution at -20°C³⁾. Chicken cardiac myofibrils were prepared by the method of Solaro *et al.*¹¹⁾.

Preparation of troponin and troponin T-Troponin was prepared from rabbit skeletal muscles according to the method of Ebashi et al.²⁾. Troponin T was separated from troponin by CM-Toyopearl (650 M) column chromatography. Troponin was applied to the CM-Toyopearl (650 M) column equilibrated with a solution containing 6 M urea, 20 mM Tris-HCl (pH 8.0), 4 mM EGTA, 80 mM NaCl and 7 mM β-mercaptoethanol. Troponin T was then eluted by increasing the NaCl concentration to 140 mM⁹⁾. Troponin T₁ was prepared from the chymotryptic digest of troponin T as described previously⁷⁾¹²⁾.

Abbreviations: EGTA, ethylene glycol bis (β-aminoethylether)-N, N, N', N'-tetraacetic acid; MOPS, 3-(N-morpholino) propanesulfonic acid; ALD, chicken anterior latissimus dorsi muscle.

The treatment of myofibrils by troponin, troponin T or troponin T₁

A standard reaction mixture contained 0.4 mg/ml myofibrils, 240 mM KCl, 5 mM MgCl₂, 20 mM MOPS (pH 6.2), 5 mM EGTA, 14 mM β -mercaptoethanol, 0.1 μ g/ml pepstatin A and 0.1 mg/ml troponin, 0.05 mg/ml troponin T or 0.03 mg/ml troponin T₁⁹. The reaction mixture was gently shaken for 60 min at 25°C and spun for 10 min at 2,500 rpm. The pellet was washed with a solution containing 150 mM KCl, 5 mM MgCl₂, 20 mM MOPS (pH 6.8), 14 mM β -mercaptoethanol and 0.1 μ g/ml pepstatin A. The resulting sediments were then used for the gel electrophoretic study and the ATPase assay.

Gel electrophoresis

SDS-gel electrophoresis was carried out by the procedure of Laemmli at a 12% acrylamide concentration⁶. The gels were stained with Coomassie brilliant blue R-250 and scanned with a dualwavelength chromatoscanner at a wavelength of 554 nm (CS-9000, Shimadzu). For the quantitative analysis of troponin T, the density of troponin T was normalized by the density of the myosin light chain 3 in each myofibril.

ATPase assay

The reaction mixture (2 ml) for the ATPase assay consisted of 90 mM KCl, 5 mM MgCl₂, 20 mM MOPS (pH 6.8), 1 mM Ca²⁺ EGTA, 2 mM ATP and 0.2 mg/ml myofibrils after treatment. The reaction was started at 25°C by the addition of ATP and terminated after 5 min by the addition of 1.5 ml of ice cold 20% trichloroacetic acid containing 4% ascorbic acid. The liberated inorganic phosphate was measured by the colorimetric method of Baginski et al.¹¹. The free Ca²⁺ concentration was calculated as described previously^{4,6}.

Protein concentration

The protein concentration was determined by the biuret reaction using bovine serum albumin

as a standard.

Results and Discussion

Fig. 1 shows the densitometric profiles of the SDS-gel electrophoretic patterns of the bullfrog skeletal myofibrils. After treatment with rabbit skeletal troponin T and troponin T₁, a density decrease was observed for all of the troponin components of the bullfrog skeletal myofibrils. After troponin T-treatment, the density at the position of tropomyosin was increased by the incorporation of rabbit skeletal troponin T, but the tropomyosin density did not significantly change after the troponin T₁ treatment. After treatment with the rabbit skeletal troponin C•I•T complex, the amount of troponin T of the bullfrog myofibrils definitely decreased while, instead, a density increase at the position of tropomyosin and rabbit troponin T was also observed on the gel (Fig. 1-b). The density at the position of bullfrog troponin I and C slightly increased after rabbit skeletal troponin treatment most likely due to the incorporation of rabbit skeletal troponin I and C into the myofibrils. This was consistent with the previous finding in which the troponin C•I•T complex in myofibrils was replaced with the added rabbit skeletal troponin C•I•T complex⁹.

The position of bullfrog troponin T on the gels could be definitely distinguished from those of other protein components and hence the change in the amount of troponin T was most clearly identified in the gels. Thus the quantitative analysis of troponin-replacement under various conditions was carried out by measuring the troponin T-density. The troponin T-content in the myofibrils decreased as the amount of added troponin, troponin T or troponin T₁ was increased (Table I). When the rabbit skeletal troponin, troponin T or troponin T₁ at an amount 1.5 times that of those proteins in the myofibrils were then added to the incubating solution, and about 50% of myofibrillar troponin T was replaced by the added rabbit

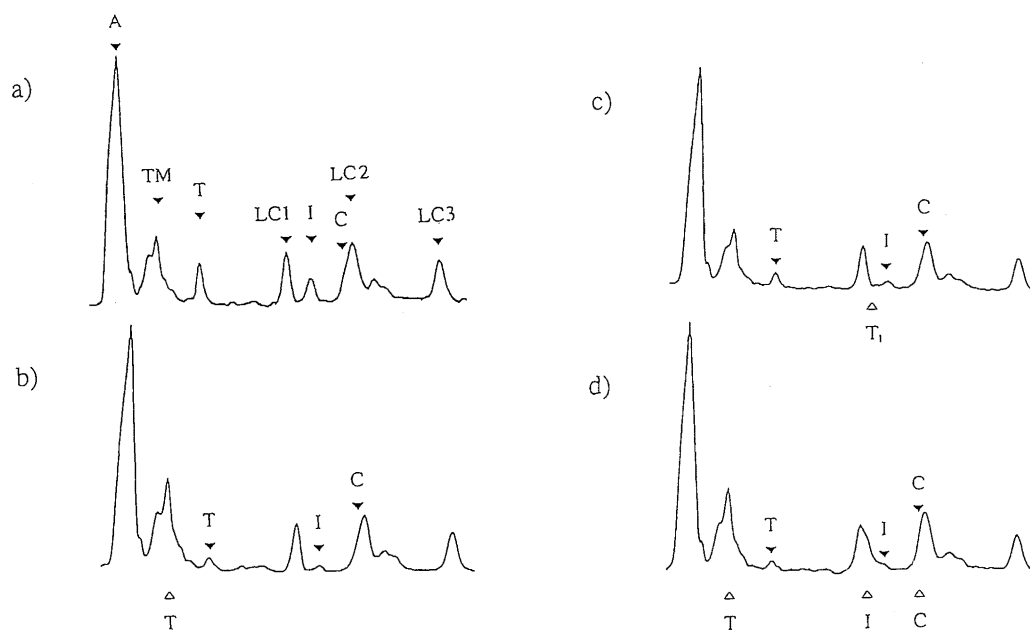


Fig. 1 Densitometric profiles of the SDS-gel electrophoretic patterns of the bullfrog skeletal myofibrils.

- a) control
- b) after troponin-treatment
- c) after troponin T-treatment
- d) after troponin T₁-treatment

The ratios of the amounts of the added proteins (rabbit skeletal troponin, troponin T or troponin T₁) to the corresponding proteins in the bullfrog myofibrils were 5.9.

Abbreviations: A, actin; TM, tropomyosin; LC1, 2 and 3, myosin light chain 1, 2 and 3; T, I and C, troponin T, I and C. The position of the endogenous troponin components are marked by closed triangles (▼), while the position of the incorporated rabbit skeletal troponin components are designated by open triangles (Δ).

Table I The effect of treatment with rabbit skeletal troponin T, troponin T₁ and troponin on the troponin T content in bullfrog skeletal myofibrils¹⁾.

Condition	Relative amount of troponin T in myofibrils (%)			
	Control	added troponin troponin T or troponin T ₁ / troponin, troponin T ₁ in the myofibrils		
		1.5	3.0	5.9
1) myofibrils after troponin T treatment	100	49	38	30
2) myofibrils after troponin T ₁ treatment	100	77	68	45
3) myofibrils after troponin treatment	100	56	29	17

1) Incubation period was 60 min at 25°C

troponin or troponin T, while about 20% of troponin T was replaced by the added troponin T₁ at the same condition. Thus, rabbit skeletal troponin T₁, a soluble chymotryptic fragment of troponin T, was less effective than troponin T or troponin in replacing the endogenous troponin complex in bullfrog myofibrils. Although about 80% of troponin T was maximally removed as the amount of added troponin increased, the extent of troponin T-removal did not exceed 70% even after treatment with an excess amount of rabbit skeletal troponin T. This was, at least partly, due to the limited amount of available troponin T solubilized in the incubating solution, because of the relatively insoluble nature of the troponin T at this low ionic strength condition⁹. The troponin T removal was also affected by the pH and KCl concentration. About 30% of the troponin T was removed at 30 mM KCl but 80% of the troponin T was constantly removed in the KCl concentration range between 90 and 240 mM (data not shown). Troponin T was removed by troponin treatment at a neutral pH (6.8-7.4) to some extent, but the amount of troponin T removed was definitely enhanced by lowering the pH to 6.2 but was not further enhanced by lowering the pH to 5.6 (data not shown). The presence of EGTA (5 mM) or ATPyS (5 mM) in the incubating solution did not affect the extent of troponin T removal

(data not shown).

The ATPase of the intact bullfrog skeletal myofibrils was inhibited at low Ca (pCa 8.2) but was activated at a high Ca (pCa 4.4). After treatment with rabbit skeletal troponin, the myofibrillar ATPase showed essentially the same Ca²⁺-activation profiles as those of the intact myofibrils in terms of maximum activation, and the Hill coefficient and pCa at half maximum activation, as shown previously¹⁰. After the treatment with rabbit skeletal troponin T, however, the ATPase was desensitized to Ca²⁺-concentrations and hence was activated at both low and high Ca²⁺-concentrations (Table II-2). This was due to the removal of both troponin I and C from the myofibrils, for the Ca²⁺-sensitivity was observed to recover after the addition of rabbit skeletal troponin C·I (Table II-3).

The myofibrillar ATPase was also desensitized to Ca²⁺ to some extent, by treatment with troponin T₁, but the Ca²⁺-sensitivity did not recover by the further addition of troponin C and I (Table II-5).

The effect of treatment with troponin T and T₁, was also examined, using the myofibrillar preparations from rabbit skeletal and various chicken muscles (Table III). The change in the troponin C content was measured as an index of the troponin substitution, for troponin I and T coincided with other myofibrillar proteins on

Table II The effect of treatment with rabbit skeletal troponin T or T₁ and the successive reconstitution with rabbit skeletal troponin C·I on the Ca²⁺-sensitive ATPase activity in the bullfrog skeletal myofibrils.

Conditions	ATPase ($\mu\text{mole P}_1 \cdot \text{mg}^{-1} \cdot \text{min}^{-1}$)		Ca ²⁺ -sensitivity [$\frac{(b)-(a)}{(a)}$]
	pCa 8.2 (a)	pCa 4.4 (b)	
1) control	0.134	0.216	0.61
2) troponin T-treated	0.216	0.225	0.04
3) troponin T-treated +troponin C·I	0.161	0.279	0.73
4) troponin T ₁ -treated	0.189	0.212	0.12
5) troponin T ₁ -treated +troponin C·I	0.201	0.229	0.14

Table III Change in the content of troponin C in various chicken myofibrils after treatment with rabbit skeletal troponin T and troponin T₁

species of myofibrils	Relative amount of troponin C in myofibrils (%)					
	Control	added troponin T		added troponin T ₁		
		troponin T in myofibril		troponin T ₁ in myofibril		
		2.5	5.0	2.5	5.0	12
A. rabbit fast skeletal	100	—	33	—	76	56
B. chicken						
1) breast fast skeletal	100	51	30	86	72	—
2) leg fast skeletal	100	42	29	76	70	—
3) cardiac	100	50	34	85	73	—
4) ALD slow skeletal	100	38	33	80	70	—

the gel (data not shown). Much more amount of troponin C was removed from all kinds of myofibrils by troponin T than by troponin T₁. Any significant difference in the amount of removal of troponin C by the treatment with either troponin T or troponin T₁ could not be observed among these five kinds of myofibrils.

In our previous paper, we showed that troponin (troponin C·I·T complex) in bullfrog skeletal myofibrils was replaced by the addition of an excess amount of rabbit skeletal troponin or troponin T (tropomyosin binding component of troponin), but was not replaced by the excessive rabbit skeletal troponin C·I⁹⁾. This indicated that the added troponin C·I·T complex binds, through troponin T, to tropomyosin in the myofibrils, while the endogenous troponin was instead expelled from the myofibrils.

Troponin T₁ is a soluble chymotryptic fragment of the amino-terminal 158 residues of troponin T of 259 amino acid residues. This fragment binds strongly to tropomyosin but not to troponin C or I and was shown not to have a Ca-regulating ability⁷⁾⁸⁾¹²⁾. On the other hand, the carboxyl-terminal side fragment of 101 residues, termed troponin T₂, binds to both tropomyosin and troponin I·C and preserves the Ca²⁺-regulating action of troponin T⁷⁾⁸⁾¹²⁾. Since the troponin replacing activity of troponin T₁ was definitely lower than that of troponin T (Table II, Table III)¹⁰⁾, the binding of

the troponin T₂ region to tropomyosin, in addition to the binding of troponin T₁ region to tropomyosin, is therefore considered to be also involved in the replacement process of troponin in myofibrils.

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(和文抄録)

ウシガエルおよびニワトリ筋原繊維内トロポニンの ウサギ骨格筋トロポニン, トロポニン T, および トロポニン T_1 による置換

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ウシガエル骨格筋筋原繊維内トロポニンのウサギ骨格筋トロポニン, トロポニン T, およびトロポニン T_1 , による置換を SDS-電気泳動上密度測定による検討した。筋原繊維内のトロポニンは, 外部から添加したトロポニン T とトロポニンによりほぼ同程度置換された。これに対して, トロポニン T の N-端側クラグメントであるトロポニン T_1 は筋原繊維のトロポニン置換をしたものの, トロポニン T に比べると置換能は

低いことが判明した。またトロポニン T_1 のトロポニン置換能は, ウサギ骨格筋, ニワトリ骨格筋(速筋, 遅筋)および, ニワトリ心筋から調製した筋原繊維標本についても, トロポニン T より低いことがわかった。これらの所見は筋原繊維内トロポニン置換には, トロポニン T 分子の N-端側の T_1 領域だけでなく, C-端側の T_2 領域も関与することを示している。