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Troponin-like regulation in muscle thin filaments of the mussel *Crenomytilus grayanus* (Bivalvia: Mytiloida)

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ABSTRACT

Muscles of bivalve molluscs have double calcium regulation — myosin-linked and actin-linked. While the mechanism of myosin-linked regulation is sufficiently studied, there is still no consensus on the mechanism of actin-linked regulation. Earlier we showed a high degree of Ca^{2+}-sensitivity of thin filaments from the adductor muscle of the mussel *Crenomytilus grayanus* (Mytiloida). In order to elucidate the nature of this regulation, we isolated the fraction of minor proteins from the mussel thin filaments, which confers Ca^{2+}-sensitivity to reconstituted actomyosin–tropomyosin. Proteins of this fraction, ABP-19, ABP-20, and ABP-28, were chromatographically purified and identified. According to the results of mass spectrometry and Western blot analysis, as well as by their functional properties, these mussel actin-binding proteins appeared to correspond to the troponin components from the skeletal muscles of vertebrates (TnC, TnI and TnT). The reconstituted mussel troponin complex confers to actomyosin–tropomyosin more than 80% Ca^{2+}-sensitivity. The in vivo molar ratio of actin/tropomyosin/troponin was calculated to be 7:1:0.5, i.e., the content of troponin in mussel thin filaments is two times lower than in thin filaments of skeletal muscles of vertebrates. These data demonstrate that troponin-like regulation found in the catch muscle of the mussel *C. grayanus* is present at least in two suborders of bivalves: Pectinoida and Mytiloida.

1. Introduction

Muscle contraction is regulated by calcium ions through thick filaments and/or through thin filaments [1]. In the case of actin-linked regulation, Ca^{2+} binds to troponin located on thin filaments; as a result, thin filaments acquire the ability to interact with thick filaments. In the case of myosin-linked regulation, Ca^{2+} binds to myosin or activates kinase, phosphorylating myosin; as a result, myosin acquires the ability to interact with thin filaments.

Muscles with myosin-linked regulation have also actin-linked regulation [2]. It is believed that in muscles with dual regulation, actin-linked regulation can be involved in the functions unique to the smooth muscle, in particular the phenomenon of the latch state in vertebrates [3,4] or the catch state in molluscs [5–7]. The smooth muscle of bivalves has a unique ability to be not in two, but in three states, namely relaxed, contracted and catch states. According to the generally accepted hypothesis, the catch is based on passive cross-links between the thick and thin filaments that are formed by twitchin and are regulated by twitchin phosphorylation [5,8].

The formation of twitchin linkages leads to "freezing" of tropomyosin in the blocked position [9]. It seems that the mechanisms of actin-linked regulation and the catch state can be functionally linked and complement each other. Myorod [6] and calponin [10], whose function is still unknown, also interact with thin filaments. Thus, thin filaments of bivalve smooth muscle represent a well-organized complex structure, which participates not only in muscle contraction, but also maintains the catch state and performs actin-linked regulation. The aim of the current study is to clarify the mechanism of actin-linked regulation of the adductor muscle of the mussel *Crenomytilus grayanus* in order to subsequently determine the role of other thin filament proteins.

2. Materials and methods

2.1. Protein isolation

The source of proteins in this study was the posterior adductor of the sea mussel *C. grayanus*, as well as the muscles of the back and hind limbs of rabbit. Mussel thin filaments, mussel "natural" F-actin (the filamentous form of actin isolated from the muscle) and mussel tropomyosin were prepared as described previously [5,10,11]. Rabbit skeletal muscle myosin was prepared according to Margossian and Lowey [12]. Rabbit skeletal muscle tissues were donated from the vivarium of the G.B.

Abbreviations: A, actin; TM, tropomyosin; Tn, troponin; TnI, component of troponin, which inhibits actin–myosin interaction; TnT, tropomyosin-binding component of troponin; TnC, calcium-binding component; ABP, actin-binding protein; DBC, dye-binding constant.

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Elyakov Pacific Institute of Bioorganic Chemistry (Vladivostok). All procedures were approved by the Animal Care Committee of A.V. Zhirmunsky Institute of Marine Biology, Far East Branch of the Russian Academy of Sciences (Protocol N 21 from 08.09.2014).

2.2. Mussel myofibrils' preparation

All procedures were done on ice. 100 g of fresh minced muscle was rigorized for 24 h with 4 volumes of glycerol solution (50% glycerol, 75 mM NaCl, 2 mM $MgCl_2$, 2 mM EGTA, 3 mM NaN_3, 0.1 mg/ml trypsin inhibitor, 0.5 mM PMSF, 1 mM DTT, 20 mM imidazole–HCl, pH 6.5) with constant agitation with an overhead stirrer. Upon completion, glycerinated muscles were ground in a meat grinder and homogenized with Polytron PT 2500E at 5000 rpm for 10 min in 1 l of washing solution containing 75 mM KCl, 1 mM $MgCl_2$, 0.2 mM EGTA, 1 mM NaN_3, 0.5 mM DTT, 10 mM phosphate buffer, pH 6.5. The homogenate was diluted up to 4 l with the washing solution and centrifuged at 5000 rpm for 20 min. The pellet was resuspended in 2 l of washing solution and recentrifuged as above. The procedure was repeated with 1 l of washing solution and the pellet was used as crude "myofibrils".

2.3. Isolation of the fraction, conferring Ca^{2+}-sensitivity to actomyosin

The fraction was isolated from either Ca^{2+}-sensitive filaments or "myofibrils". In both cases, the fraction conferred Ca^{2+}-sensitivity to actomyosin; this indicates that the desired regulatory proteins are localized in thin filaments. Isolation of this fraction from "myofibrils" is described below.

The last washing pellet («myofibrils») was suspended in 1 l of washing solution, where 20 mM imidazole–HCl, pH 6.5 buffer was substituted with 50 mM citrate buffer, pH 4.9. The suspension was centrifuged as above; 200 ml washing solution (pH 4.9) and 300 ml of double LiCl-extracting solution (0.8 M LiCl, 1 mM EGTA, 6 mM NaN_3, 2 mM DTT, 1 mM PMSF, 50 mM citrate buffer, pH 4.9) were added to the residue. The extraction was conducted for one hour with stirring. It was followed centrifugation at 10,000 rpm for 20 min (Beckman rotor F0685). The extract was clarified by centrifugation at 40,000 rpm for 30 min (Beckman rotor type 50.2). The clarified extract was fractionated by salting out with solid ammonium sulfate of 20–70% saturation. The fraction was collected by centrifugation, dissolved in 20 ml of dialyzing solution containing 75 mM KCl, 2 mM $MgCl_2$, 0.2 mM EGTA, 2 mM NaN_3, 0.5 mM DTT, and 25 mM imidazole HCl (pH 6.5) and dialyzed against this solution. The dialyzed fraction was clarified by centrifugation at 40,000 rpm for 30 min (Beckman rotor type 50.2).

2.4. Isolation of actin-binding proteins: ABP-19, ABP-20, and ABP-28

The fraction, conferring Ca^{2+}-regulation to actomyosin was dialyzed against three changes of 20 volumes of column buffer (6 M urea, 30 mM KCl, 1 mM EGTA, 2 mM NaN_3, 0.5 mM DTT, and 10 mM Tris–HCL, pH 7.6). The dialyzed fraction was applied on a DEAE-Sepharose CL6B (Sigma-Aldrich) column (1 × 25 cm), equilibrated with the same buffer. The fraction proteins, ABP-19, ABP-20 and ABP-28, were absorbed on DEAE-Sepharose, while actin contamination passed through (Fig. 1A). The column was eluted in two steps, with a linear KCl gradient from 0.03 to 0.2 M KCl and from 0.2 to 0.35 M KCl. ABP-19 was eluted in a single peak at about 0.35 M KCl. ABP-28 was eluted together with the ABP-20 component at about 0.1–0.2 M KCl. In order to separate ABP-28 and ABP-20 proteins, we used CM Sephadex C-50 (Sigma) for cation-exchange chromatography (Fig. 1B). The fractions, containing ABP-28 and ABP-20, were dialyzed against the column buffer and were applied on a CM Sephadex column (1 × 25 cm), equilibrated with the same buffer. The column was eluted, like a previous one, in two steps. ABP-28 was eluted with a linear KCl gradient from 0.03–0.175 M, ABP-20 with a linear KCl gradient from 0.175–0.5 M KCl.

ABP-19 was dialyzed against the solution containing 75 mM KCl, 2 mM $MgCl_2$, 0.2 mM EGTA, 2 mM NaN_3, 0.5 mM DTT, and 25 mM imidazole HCl (pH 6.5). ABP-20 and ABP-28 were dialyzed against 150 mM KCl, 1 mM $MgCl_2$, 0.2 mM EGTA, 2 mM NaN_3, 0.5 mM DTT, 0.5 mM PMSF, 20 mM Tris–HCl pH 7.0, because of insolubility in a low ionic strength medium.

Reconstruction of mussel troponin complex was performed by mixing components in the column buffer in an equimolar ratio, followed by day-long dialyze against the dialyzing solution.

2.5. Western blot analysis

In order to perform Western blot analysis, a mixture of troponin components (1:1:1 in moles) was separated by SDS PAGE. Thereafter, electrophoresis gel was used for Western blot as described previously [10]. The membranes were probed with polyclonal antibodies that recognize human skeletal muscle troponin I (Novus biologicals, NB200-432; 1:1000), human skeletal troponin T (Novus biologicals, H00007138-B01P; 1:1000) and human cardiac troponin C (Novus biologicals, H00007134-A01; 1:500).

2.6. Mass spectrometry

For matrix-assisted laser desorption/ionization time-of-flight tandem mass spectrometry (MALDI/MS–MS), ABP-19, ABP-20 and ABP-28 were separated by SDS-PAGE and protein bands, after staining with Coomassie Blue R-250, were manually excised from the gel and subjected to in-gel digestion procedure with the trypsin solution (6 ng/μl of trypsin sequencing grade from Promega). Mass spectrometry analysis of the samples was carried out on the Easy-nLC II system (Proxeon Biosystems, Odense, Denmark) and coupled to the LTQ Orbitrap Velos (ThermoFisher Scientific, Bremen, Germany) equipped with a Proxeon nanoelectrospray ion source. MS/MS-spectra were matched with a

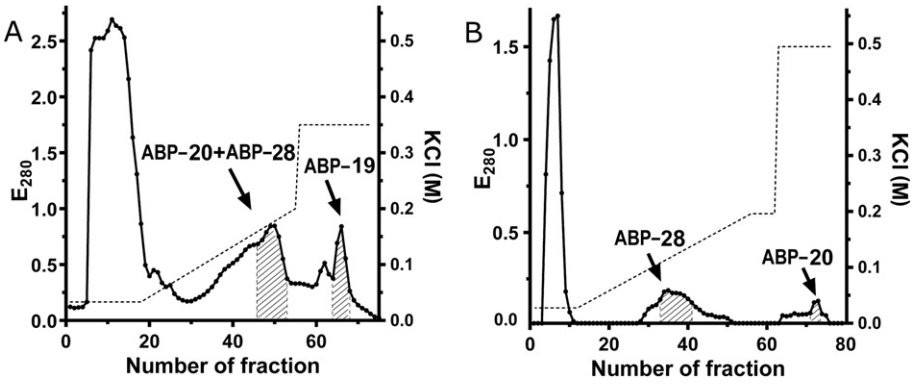

Fig. 1. Isolation of actin-binding proteins from the Ca^{2+}-sensitive fraction with the use of anion (A) and cation (B) ion-exchange columns. Details are given in "Materials and methods".