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Is nebulin truly a component of the thin filament?

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Abstract **Thin filaments were prepared from rabbit and beef skeletal muscle with three different procedures, both at high and low ionic strength. Nebulin was always found to be associated with the myosin fraction and was always absent from the thin filament fraction.**

Key words: Nebulin; Thin filament; Deuterium oxide

1. Introduction

Nebulin is an inextensible full-length molecular filament that is coextensive with thin filament in skeletal muscle [1–3] and, in different species, varies in size in proportion to thin filament length [4]. It was also reported that all nebulin subfragments bind with a specific location to the I-band in situ [5] and that cloned nebulin fragments cosediment with F-actin and bind to F-actin in the solid-phase binding assay [6]. Furthermore it was shown that rhodamine–phalloidin binds to thin filaments starting from the pointed end and promotes the redistribution of the fluorescent bands associated with the epitopes of bound nebulin antibody into a single band located close to the Z-line [7]. Taken together these evidences seem to indicate that nebulin may be a component of the thin filament. An ultimative confirmation of this point of view was apparently provided by the observation that α-actinin and nebulin cosediment with thin filaments [8].

We have now prepared thin filaments from rabbit and beef skeletal muscle, both at high and low ionic strength, and have always found nebulin to be present in the myosin fraction and absent from the thin filaments fraction. We propose therefore that, even if nebulin binds to thin filaments, the binding is very weak.

2. Materials and methods

All purification procedures were performed at 4°C. Thin filaments were prepared according to the following procedures.

2.1. Extraction of myofibrils at high ionic strength

Glycerinated rabbit psoas muscle fibers were first prepared according to Szent-Gyorgyi [9] as modified by Huxley and Hanson [10]. From this preparation myofibrils were isolated according to Huxley [11]. Myofibrils were then suspended in 100 vol. of a solution containing 0.6 M NaCl, 0.01 M pyrophosphate, 0.1 M orthophosphate, 1 mM $MgCl_2$, pH 6.4, and extracted for 90 min. The suspension was then centrifuged for 10 min at $6500 \times g$. The supernatant solution (containing myosin; Fig. 1, lane 7) was collected. The pellet was extracted for 20 min with 10 vol. of the same solution and centrifuged for 10 min at $6500 \times g$. The thin filament pellet (Fig. 1, lane 6) was suspended in 2 vol. of a solution containing 0.1 M KCl, 5 mM orthophosphate, 3 mM $MgCl_2$, 3 mM EDTA, pH 7.0 [10].

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2.2. Extraction of myofibrils at pH 6.0 and moderate ionic strength

Myofibrils, prepared according to Huxley [11], were homogenized 4×15 s at 10 s intervals in a Sorvall Omnimixer at maximum speed setting, in a solution containing 0.5 mg of myofibrils per ml, 0.1 M KCl, 5 mM orthophosphate, 3 mM $MgCl_2$, 3 mM EDTA, 5 mM ATP, pH 7.0. After homogenization the remainig myofibrils were removed by centrifugation for 10 min at $5000 \times g$. The supernatant solution was brought to pH 6.0 with 1 M HCl and centrifuged for 50 min at $30\,000 \times g$, followed by a further centrifugation for 30 min at $50\,000 \times g$. The two pellets, containing the thick filaments, were collected and suspended in 0.3 M NaCl. The supernatant solution was centrifuged for 3 h at $100\,000 \times g$. The pellet, containing the thin filaments, was suspended in a solution containing 50 mM KCl and 10 mM Tris-HCl, pH 7.0 [12].

2.3. Extraction of myofibrils at low ionic strength

'Natural' actomyosin was prepared from beef muscle according to the procedure of Meng et al. [8]. Minced beef skeletal muscle (50 g) was mixed with 3 vol. of 10 mM NaOH, 1 mM (final concentration) phenylmethyl sulfonyl fluoride, 10 mg/ml (final concentration) casein and homogenized for 2 min in a Waring Blendor (Fig. 2, lane 1). The homogenate was centrifuged for 10 min at $17\,000 \times g$. The pellet was homogenized again 4×1 min at 10 min intervals (Fig. 2, lane 2). The pH was adjusted to 6.8 by addition of 1 M HCl and the suspension (150 ml) was dialyzed overnight against 1000 ml of a solution containing 5 mM ATP, 8 mM $MgCl_2$, 1 mM EGTA, 1 mM phenylmethyl sulfonyl fluoride, pH 7.0. The dialysate was then centrifuged for 10 min at $12\,000 \times g$ to collect myosin (Fig. 2, lane 4) and the supernatant solution (Fig. 2, lane 3) was centrifuged at $360\,000 \times g$ for 10 min to collect the thin filaments (Fig. 2, lane 5).

Dodecyl sulfate-polyacrylamide gel electrophoresis (PAGE) was performed as described by Wang [13]. Both 3.2% and 4% polyacrylamide slab gels were prepared using acrylamide/bisacrylamide (50 : 1).

Protein was determined by the Coomassie blue method [14] as modified by Stoscheck [15]; bovine serum albumin was used as a standard. The approximate concentration of myofibrils was determined by dissolving them in 2% sodium dodecyl sulfate (SDS) and measuring the absorbance at 280 nm ($A^{1\%}_{280} \sim 7$) [15].

3. Results

The composition of the thin filament pellet prepared from myofibrils extracted at high ionic strength (Section 2.1) is presented in Fig. 1. Nebulin is clearly detected in lanes 3 (glycerinated muscle fibers), 5 (mixture of thin and thick filaments) and 7 (thick filaments) but it is not detectable in lane 6 (thin filaments).

On the assumption that nebulin could have been dissociated from thin filaments because of treatment at high ionic strength, a different procedure (Section 2.2), involving the use of low ionic strength media, was essayed. Also in this case, however, at the end of the purification procedure, nebulin was found in the fraction containing the thick filaments and was absent from the fraction containing the thin filaments (data not shown).

Recently nebulin was reported to cosediment with thin filaments prepared from 'natural' actomyosin [8]. We tested this possibility by ourselves isolating 'natural' actomyosin [8] and preparing thick and thin filaments also from this source. Dis-

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sociation of thin and thick filaments was achieved by dialysing overnight 'natural' actomyosin against a low ionic strength medium (5 mM ATP, 8 mM $MgCl_2$, 1 mM EGTA, 1 mM phenylmethyl sulfonyl fluoride, pH 7.0) (Section 2.3). At the end of the dialysis thin and thick filament appeared to be cleanly separated, as it is shown by PAGE, and nebulin was found to be present exclusively in the thick filaments fraction (Fig. 2, lane 4) and to be absent from the thin filaments fraction (Fig. 2, lanes 3 and 5).

4. Discussion

Convincing evidence has been so far obtained that nebulin is an inextensible filamentous protein running parallel to the thin filament from the Z-line toward the center of the sarcomere [1–5].

On the contrary, cosedimentation and binding in solid-phase assay of cloned nebulin fragments to F-actin in vitro cannot be taken as a proof of nebulin being a component of the thin filament. Dozens of proteins were found to interact with F-actin in vitro but only a few of these complexes are of physiological significance. Furthermore, it cannot be excluded that adventitious F-actin binding sites, absent from native nebulin, are generated in the nebulin fragments.

The observation that rhodamine–phalloidin induces the redistribution of the fluorescent bands associated with the epitopes of bound nebulin antibodies into a single band close to the Z-line [7] could indicate a thin filament–nebulin interaction. Also in this case, however, interpretation of the data is not a simple matter since phalloidin also binds to the Z-line with complex effects on muscle tension [17,18]. Thus, the redistribution of nebulin epitopes could recognize a different cause than labelling the thin filament with phalloidin.

The report that nebulin and thin filaments, prepared from 'natural' actomyosin, cosediment could be taken as the final proof that nebulin is indeed a component of the thin filament [8]. Unfortunately, all our attempts to reproduce these data were unsuccessful: nebulin was always present in the myosin fraction and was always absent from the thin filament fraction.

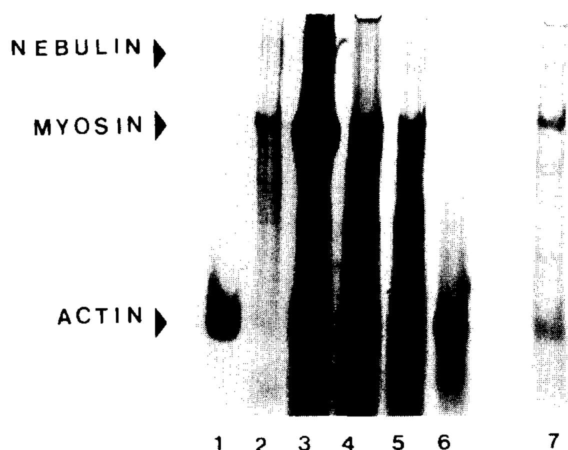

Fig. 1. 4% SDS-PAGE of thin filaments isolated at high ionic strength. Lane 1, actin (40 μg); Lane 2, myosin (40 μg); Lane 3, glycerinated muscle fibers (150 μg); Lane 4, myofibrils first pellet (200 μg); Lane 5, mixture of thin and thick filaments (200 μg); Lane 6, thin filaments (260 μg); Lane 7, thick filaments (280 μg). Electrophoresis was run at 22°C in 40 mM Tris-acetate, 20 mM sodium acetate, 2 mM EDTA, 0.1% (w/w) SDS, pH 7.4, at 5 mA for the first 2 h and at 50 mA for the remaining 4 h.

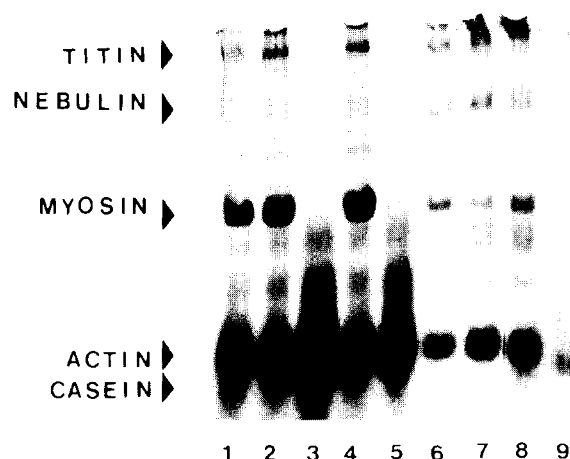

Fig. 2. 3.2% SDS-PAGE of thin filaments isolated at low ionic strength from 'natural' actomyosin. Lane 1, muscle fibers after the first homogenization; Line 2, muscle fibers after the second homogenization; Lane 3, supernatant solution after dialysis (thin filaments); Lane 4, pellet after dialysis (thick filaments); Lane 5, pellet of thin filaments; Lanes 6–8 standard samples; Lane 9, casein. Electrophoresis was run at 22°C in 40 mM Tris-HCl, 20 mM sodium acetate, 2 mM EDTA, 10 mM 2-mercaptoethanol, 0.1% (w/w) SDS, pH 8.4, at 8 mA overnight.

The discrepancy could be related to the use of deuterium oxide in the dissociation of 'natural' actomyosin. As a matter of fact, Meng et al. [8] clearly point out that the separation of thin filaments from myosin is unsatisfactory in the absence of D_2O.

Actually, D_2O significantly alters the properties of the solvent. In D_2O acid dissociation constants are usually about one-fifth those observed in H_2O [19] and the α-helical form is favoured in protein [20]. It is thus to be expected that, in D_2O, both the charge and the helical content of nebulin may change, perhaps favouring the formation of a complex with the thin filament. Due to the properties of nebulin this seems a particularly plausible hypothesis. In fact up to 97% of the nebulin sequence is assembled from repeats of a sequence motif 35 amino acid residues long and these repeats show a tendency to fold as transient helices in aqueous solutions [21,22].

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