THE ORIGIN OF 30,000 DALTON PROTEIN IN TROPONIN PREPARATIONS

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1. Introduction

It is already well established that the Ca^{2+} sensitivity of actomyosin is rendered by the regulatory protein system, composed of troponin and tropomyosin [1, 2]. Troponin appeared to consist of several components which can be separated by DEAE-Sephadex chromatography [3, 4]. We have recently shown [4, 5] that out of three main constituents of troponin called: TN-C (mol. wt. 18,000), TN-I (mol. wt. 24,000) and TN-B (mol. wt. 39,000) (the average molecular weights and the nomenclature were taken from [5]) two latter proteins are very susceptible to the proteolytic digestion. Thus, the other constituent of troponin corresponding to the protein of molecular weight 13,000–14,000 daltons, was found to be the product of catheptic degradation of TN-I, formed during preparation of troponin, namely during incubation at pH 4.6 [4, 5]. In addition, troponin preparations contained usually variable amounts of another protein of molecular weight of about 30,000 daltons [4, 6–8]. We have recently found particularly large amounts of this protein in troponin preparations from slow and cardiac muscles [9] concominantly with considerable decrease of TN-B. The observation suggested that 30,000 component might derive from TN-B. This assumption obtained experimental evidence in the present work.

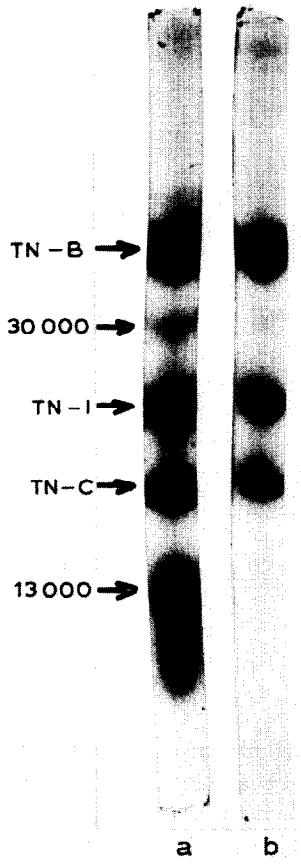

Fig. 1. SDS-polyacrylamide gel electrophoresis of troponin: a) troponin prepared from 1 M KCl extract from acohol–ether muscle powder [10]: b) troponin prepared with LiCl method [11]. Electrophoresis performed according to Weber and Osborn [12].

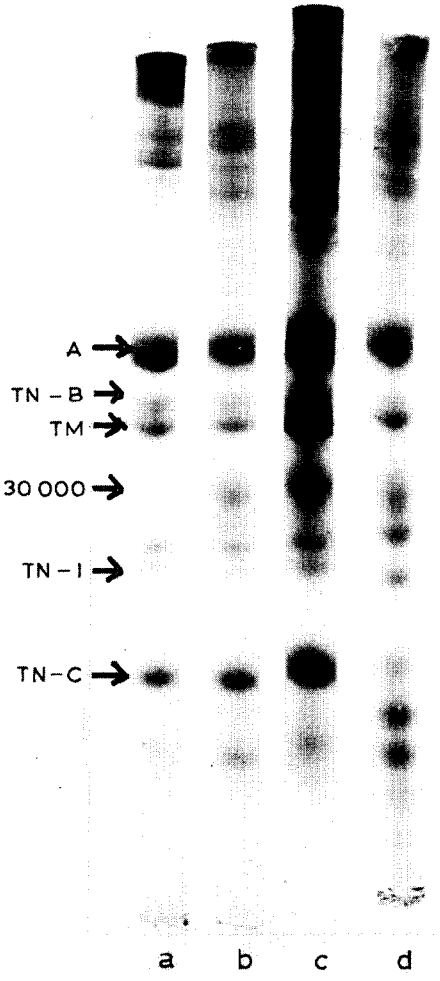

Fig. 2. SDS-polyacrylamide gel electrophoresis of rabbit myofibrils: a) fresh; b) after storage at 0° for 7 days, c) after glycerination and storage at −20°; d) after 20 min digestion with 10 μg trypsin per mg protein at room temperature (TM = tropomyosin).

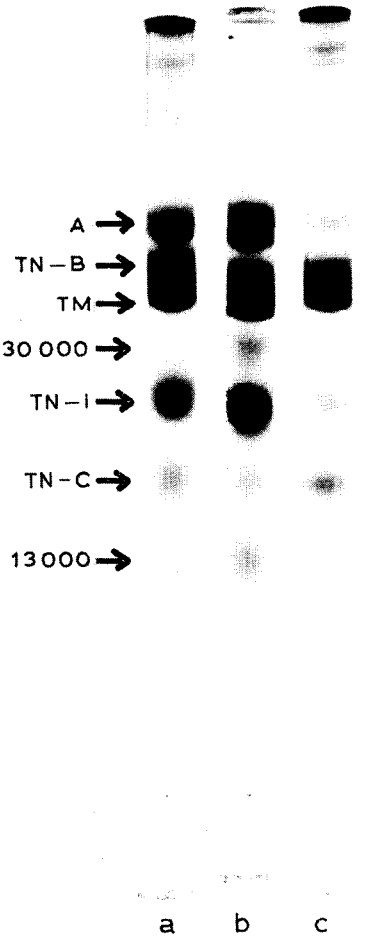

Fig. 3. SDS-polyacrylamide gel electrophoresis of 1 M KCl extract from alcohol–ether muscle powder [10]; a) after 30 min extraction at room temp.; b) after 24 hr extraction; c) before extraction the whole suspension was boiled at 100° for 5 min followed by 24 hr extraction.

2. Experimental

The typical sodium dodecyl sulphate (SDS) gel electrophoresis of troponin is shown in fig. 1. The comparison of the pattern of various troponin preparations suggested a parallelism between the decrease of the amount of TN-B and the increase of 30,000 component. The observation suggested that this protein was a product of degradation of TN-B. Since fresh myofibrils did not contain 30,000 protein (fig. 2a), the question arose on which step of preparation of troponin it was formed. Preparations of troponin based on the extraction of dehydrated muscle powder with a high ionic strength solution [10, 13] contained usually relatively high amounts of 30,000 protein. Moreover, its amount often increased during storage of troponin. As a result an almost complete lack of TN-B was sometimes concomitantly observed. Therefore this method of preparation was chosen for the investigations. Throughout the whole procedure thymol was added to all the solutions used in order to avoid bacterial contamination. When alcohol–ether powder from rabbit leg and back muscles was

extracted with 1 M KCl a 30,000 dalton band was usually seen already in the unfractionated extract. Its amount depended on the time of extraction of the muscle powder. In the first 30 min extract we did not observe the presence of 30,000 protein (fig. 3a), but after 24 hr of extraction it appeared in considerable amount (fig. 3b). As the second step of degradation the formation of fragments of molecular weight about 13,000 was observed, i.e. similar to those formed by the action of lysosomal cathepsins at acid pH [4, 5]. On the other hand, 5 min boiling of the suspension of muscle powder in 1 M KCl at the beginning of the extraction inhibited the formation of 30,000 protein (fig. 3c). The above observations showed that this protein is formed before adjustment of the 1 M KCl extract to pH 4.6 and suggested that not the lysosomal cathepsins active at acid pH range and causing degradation of TN-I to 13,000 protein [4, 5], but another protease(s) active at pH around neutral is responsible for the degradation of TN-B to 30,000 protein.

As it was shown in our laboratory [14] autolysis of muscle homogenate at neutral pH range, measured by the increase of Folin-positive material soluble in 5% trichloroacetic acid, was considerably inhibited by diisopropyl fluorophosphate (DFP). However, addition of this reagent even at 1 mM concentration to each step of preparation of troponin did not inhibit formation of 30,000 protein.

The proteolytic activity causing the formation of 30,000 protein is tightly bound to myofibrils. Thus, even well washed myofibrils after storage for several days in ice, or glycerinated myofibrils, reveal the presence of the band corresponding to 30,000 dalton protein (fig. 2b, c). Even actin after one purification cycle [15] contains not only acidic [17] but also neutral protease(s). On the electrophoretic pattern of such actin some amounts of 30,000 and 13,000 components can be usually seen (fig. 4a). These proteins derive most probably from TN-B, which in the troponin complex seems to be the component which has high affinity for actin [2, 5]. After removal of TN-B and/or its degradation products, electrophoretically pure actin (fig. 4b) can still contain traces of proteolytic activity. For instance, F-actin preparations mixed with troponin devoid of 30,000 dalton band, often reveal in the pellets obtained after sedimentation in Spinco ultracentrifuge, the presence of 30,000

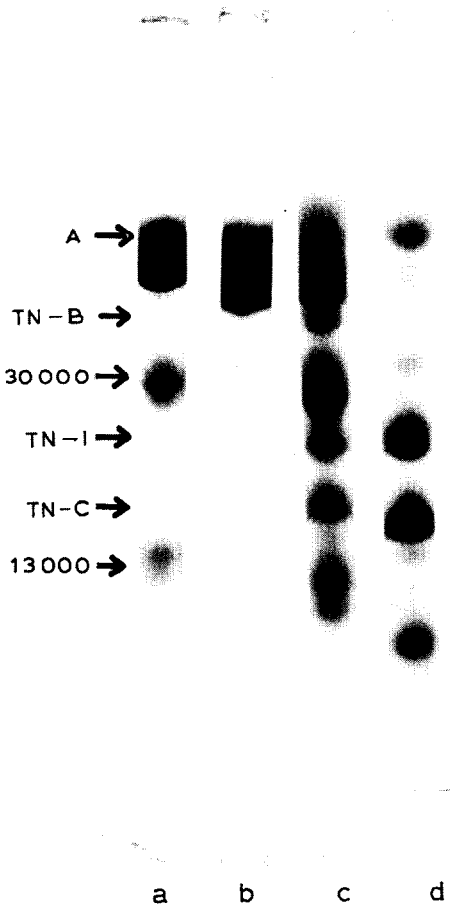

Fig. 4. SDS-polyacrylamide gel electrophoresis of actin: a) extracted at 0° and subjected to one purification cycle by polymerization in 0.1 M KCl and ultracentrifugation [15]; b) the same as in a) but after two purification cycles, including after polymerization with 0.1 M KCl an incubation at 0.5 M KCl [16]. Samples of F-actin obtained as in b) were mixed with troponin obtained with LiCl method (fig. 1b) in 1:1 weight ratio. The precipitate formed was removed by centrifugation: c) precipitate, d) supernatant (A = actin).

protein. Similarly this protein appears in the precipitates formed when preparations of troponin rich in TN-B (and devoid of 30,000 component) (fig. 1b) are added to F-actin (fig. 4b, d) [5].

Another support for the view that 30,000 component derives from TN-B is furnished by the results of mild digestion with trypsin. Our recent studies showed that the first sign of digestion of troponin is the appearance of 30,000 protein and a decrease of the

amount of TN-B [9]. Only the increase of the concentration of trypsin or of time of incubation caused gradual decline of two latter proteins, as well as of TN-I, and formation of low molecular weight (10,000 to 13,000 daltons) components [9]. Also during a very mild digestion of myofibrils 30,000 protein appears at the early stage of digestion.

Thus, the present results extend the previous observations pointing out the danger of proteolytic splitting during preparation of troponin. It is clear that the commonly used methods of preparation based on the extraction of alcohol–ether powder with the solutions of high ionic strength [10, 13] strongly favour the splitting of TN-B. Similarly, troponin obtained according to the earlier method of Ebashi et al. [1], based on the extraction of muscle residue devoid of myosin with the solution of low ionic strength, also contains 30,000 protein. In all of these methods in addition to 30,000 protein some amounts of the lower molecular weight products (about 13,000 daltons) are also formed before incubation at pH 4.6. It remains to be elucidated whether the latter degradation products are identical with those formed by the action of lysosomal cathepsins.

The present results indicate that only the method based on the extraction of fresh muscle with LiCl at pH 4.5 [11], avoiding prolonged incubation at neutral pH, yields troponin freed of any degradation products.

Our knowledge about proteolytic enzymes of mammalian skeletal muscle active at neutral pH range is very poor. At any rate this activity is rather low but, as this work points out, is sufficient to split TN-B component of troponin, which among all protein entities of myofibril seems to be the most susceptible to proteolytic splitting. Judging from the appearance of the same or very similar products, the mode of action of muscle neutral proteinase(s) is simiar to that of trypsin. On the other hand, contrary to trypsin, this activity is not inhibited by DFP, and we have not succeeded so far in finding any inhibitor preventing the splitting of TN-B.

Experiments are now in progress to elucidate whether 30,000 protein band present in considerable amounts in frog thin filaments [18] and in the troponin preparations from insect flight muscle [19] as well as from cardiac and slow rabbit muscle [9], represents the same protein as that found in rabbit fast muscle, originating in all those cases from TN-B due to the higher proteolytic activity of other types of muscle.

Acknowledgement

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