CHARACTERIZATION OF THE Ca^{2+}-REGULATORY COMPLEX OF CHICK EMBRYONIC MUSCLES: POLYMORPHISM OF TROPOMYOSIN IN ADULT AND EMBRYONIC FIBERS*

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Summary: The Ca^{2+}-regulatory tropomyosin-troponin complex was purified from chick embryonic muscles by a combination of DEAE-cellulose chromatography and $(NH_4)_2SO_4$ fractionation. The embryonic complex was very similar to that obtained from adult chicken muscles with respect to stoichiometry of components and biological activity. Tropomyosin of embryonic skeletal muscles contains both α and β subunits, the β form being the major species. In the adult stage the β form is decreased with a concomitant increase in the α form. These results indicate that i) the Ca^{2+}-regulatory proteins are not deficient in early embryonic muscles as previously thought (Hitchcock, S.E., Develop. Biol. 23, 399,1970), and ii) different structural genes coding for tropomyosin subunits are expressed differentially in embryonic and adult muscle fibers.

The major contractile proteins - myosin, Tn, TM and actin - are present in the myofibrils of adult rabbit skeletal muscles, and presumably in other vertebrate skeletal muscles, in a well defined 1:1:1:7 molar ratio (1; for a review see ref. 2). The TM-Tn complex, located in thin filaments, is responsible for Ca^{2+}-linked regulation of muscle contraction; and within this complex the molar ratio of the individual polypeptide chains - TnT, TnI, TnC and Tm - is 1:1:1:2 (1,2). An unresolved key question in myogenesis is whether or not the contractile proteins are coordinately assembled in myofilaments during all stages of muscle development. Herrmann and coworkers have shown that TM and myosin accumulate at parallel rates in chick leg muscles from day 11 to 5 months posthatching (for a review see ref. 3). Hitchcock has reported that natural AM isolated from 14-day-old muscles and earlier has a reduced Ca^{2+}- sensitivity and Ca^{2+}-binding capacity compared

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Abbreviations Used: Tn, troponin; TnT, troponin T; TnI, troponin I; TnC, troponin C; TM, tropomyosin; TM-Tn, tropomyosin-troponin; Tm, single polypeptide chain of tropomyosin; AM, actomyosin; DTT, dithiothreitol; SDS, sodium dodecyl sulfate; EGTA, ethylene glycol bis (β -aminoethylether)-N,N-tetraacetate.

to AM isolated from later stage embryos or adult muscles (4). Based on these results, she concluded that early embryonic muscle is deficient in the regulatory proteins, possibly in Tn which becomes incorporated into the AM complex late in development. Masaki and Yoshizaki (5), on the other hand, have detected in muscles of early embryos proteins which react with antibodies for myosin and Tn. Obinata et al. (6) have recently isolated from 13-day-old chick leg muscles native thin filaments whose addition to myosin produces Ca^{2+}-sensitive AM ATPase. However, in none of these studies has the TM-Tn complex been isolated and biochemically characterized. In view of these conflicting reports and as a part of our studies on the biogenesis of the Ca^{2+}-regulatory complex during muscle development, we have undertaken a detailed study of the TM-Tn complex in embryonic muscles. In this report we describe a simple and novel chromatographic method for the isolation of TM-Tn from chick embryonic muscles and its biochemical characterization. In addition, we also report a characteristic switch of the polymorphic forms of TM subunits from the embryonic to the adult stage.

METHODS

Ether dried muscle powder was prepared from 12 and 14-day-old chick leg and breast muscles by the procedure of Greaser and Gergely (7) except that the homogenizing buffer was modified to 5 mM Tris-HCl, pH 8.0; 50 mM KCl; 0.1 mM $CaCl_2$ and 0.2% Triton X-100. The isolation of TM-Tn by chromatography of the ether powder extract on DEAE-cellulose is described in the legend to Fig. 1. Rabbit skeletal myosin was prepared and purified on a column of DEAE-cellulose (8) as previously described. Actin was prepared from rabbit skeletal muscles by the method of Spudich and Watt (9). Reconstituted rabbit AM was freshly made by combining myosin and actin in a 4:1 ratio by weight. The AM was washed once with 2 mM Tris-HCl, pH 7.5 and 5 mM DTT, then twice with 2 mM Tris-HCl, pH 7.5 and 0.1 mM DTT and finally suspended in this buffer. The biological activity of embryonic TM-Tn was measured by determining the EGTA inhibition of rabbit AM ATPase in the presence of TM-Tn. Details of the assay are described in the legend to Fig. 4.

RESULTS AND DISCUSSION

Separation of TM-Tn by DEAE-cellulose chromatography: Initial attempts to prepare crude TM-Tn from embryonic muscles by conventional $(NH_4)_2SO_4$ fractionation of extracts of muscle ether powder (7) proved highly unsatisfactory in our hands, presumably due to the high nucleic acid content in embryonic muscles. It was observed that the Ca^{2+}-regulatory complex from embryonic muscles binds strongly to DEAE-cellulose and this property was utilized for the isolation of the complex. Chromatography of the extracts of ether dried muscle powder on a column of DEAE-cellulose (Fig. 1) gave three distinct peaks; an early peak eluted with column buffer, and two eluted with the KCl gradient. SDS-gel electrophoresis of the pooled fractions showed that the first peak contained the bulk of the applied proteins (Fig. 2, gels a and b). The second peak, eluted at about 0.17M KCl, showed 4 major protein bands

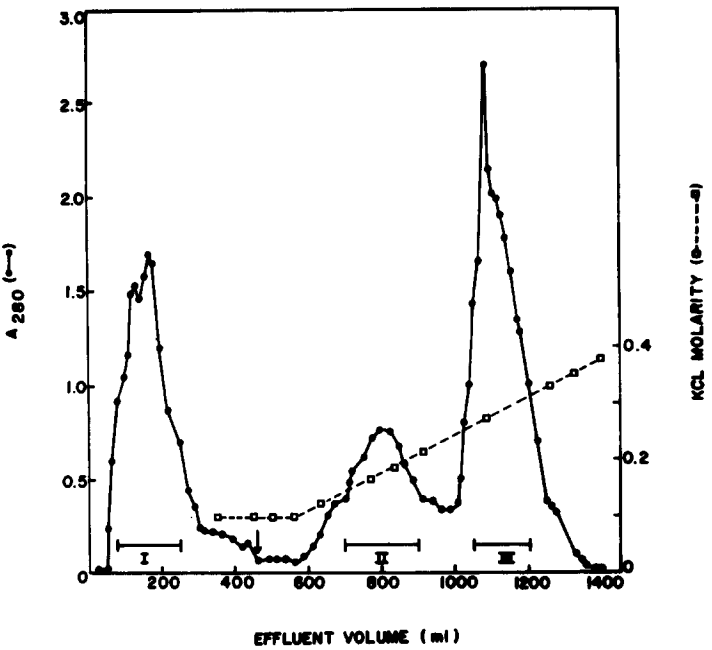

Fig. 1: Isolation of embryonic TM-Tn by DEAE-cellulose chromatography. For details see Text. Extracts of dried ether powder of embryonic leg and breast muscles were dialyzed against buffer I (0.1M KCl, 50 mM Tris-HCl, pH 8.0). About 750 mg protein was applied to a DEAE-cellulose column (25 x 2.5 cm) equilibrated with buffer I. After washing with 400 ml of buffer I, 1000 ml of a linear KCl gradient (0.1 - 0.5M in buffer I) was applied. Peak fractions indicated by the bar were pooled.

(gel c). Comparison with crude TM-Tn, prepared from adult chicken muscles, indicated that three of these bands corresponded to the known protein components of TM-Tn (gel e). The second major band is resolved in some gel runs into two separate bands of TnT and Tm (Fig. 3). The additional protein component (labeled as A) corresponded to the band of actin (see also Fig. 3). The third peak gave a few minor bands (gel d), and its high A_{260}/A_{280} ratio (1.95-2.0) suggests that it contains mainly nucleic acids. When the material present in peak II was applied to a column of Sephadex G-75, about 95% of the uv-absorbing material was eluted in the void volume (results not shown here). Electrophoresis of this fraction gave the same band pattern as gel c (cf. Fig. 2) indicating that the material was indeed a complex of TM-Tn containing actin.

Characterization of the Components of Embryonic TM-Tn: Fractionation with $(NH_4)_2SO_4$ and isoelectric precipitation (7) were used to characterize embryonic TM-Tn. When the peak fractions were adjusted to 40% saturation with $(NH_4)_2SO_4$,

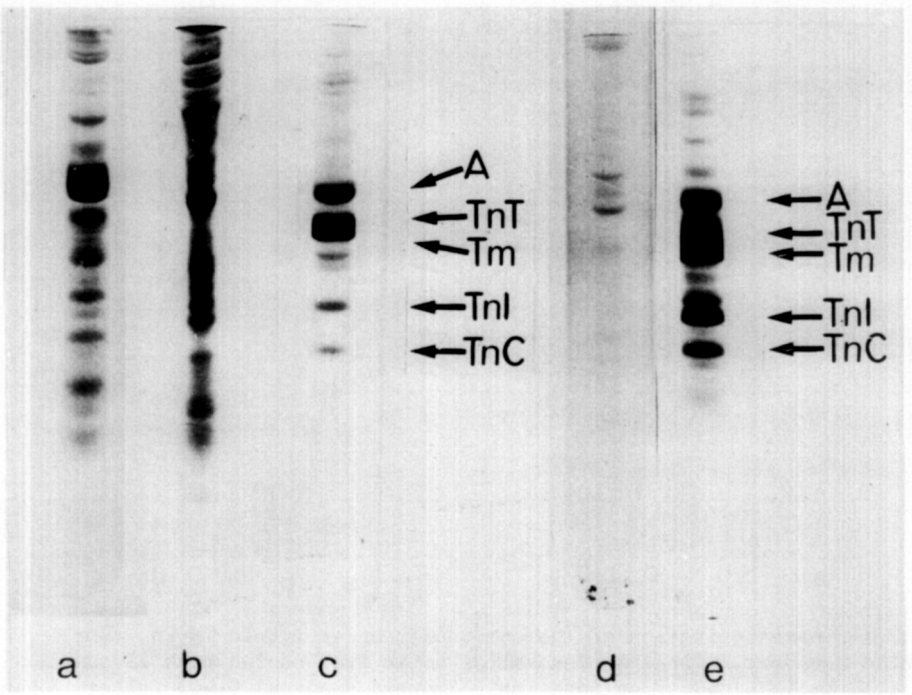

Fig. 2: SDS-gel electrophoresis of column fractions (Fig. 1). Electrophoresis was carried out on 10 cm of 10% gels (8,14). Amounts used: a) material applied to the column, 90μg; b) peak I, 80μg; c) peak II, 50μg; d) peak III, 0.1 A_{280} unit; e) adult chicken TM-Tn, 50μg.

the resulting precipitate consisted mainly of actin (Fig. 3, panel A, gel a) as shown by comparison with the purified actin run as a marker (gel c). The precipitate of the supernatant fraction obtained at 40-60% $(NH_4)_2SO_4$ saturation showed the presence of four bands (gel b) corresponding to the components of TM-Tn (see also Fig. 2) and no detectable level of actin. This was confirmed by electrophoresing a mixed sample of actin and the purified TM-Tn (gel d). The minor band between TM and TnI, which showed variable intensity in different gel runs, is probably due to a proteolytic fragment of TnI, previously reported in rabbit skeletal Tn (7). In order to separate the complex into TM and Tn, the pooled peak II fractions were precipitated at pH 4.6 and the procedure was repeated three times with both the supernatant (Tn) and precipitate (TM). The latter contained a considerable amount of actin and minor amounts of Tn components (Panel B, gel b). The final TM fraction was also subjected to $(NH_4)_2SO_4$ fractionation. The material obtained at 0-50% saturation showed a prominent band of actin (gel c), and the 50-60% $(NH_4)_2SO_4$ fraction gave actin-free TM

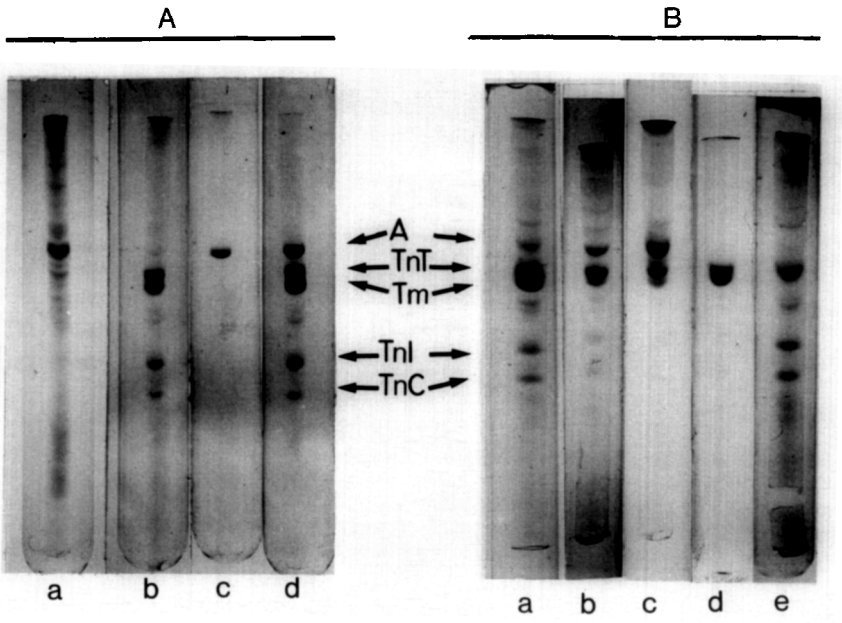

Fig. 3: Characterization of the components of embryonic TM-Tn. For details see Text. Panel A. Removal of actin from pooled peak II samples (Fig. 1) by $(NH_4)_2SO_4$ fractionation. Amounts used: a) 0-40% pellet, 12μg; b) 40-60% pellet, 50μg; c) purified chicken actin (marker), 10μg; d) mixed sample of 40-60% pellet, 50μg , and marker actin, 10μg. Panel B. Purification of TM and Tn by isoelectric precipitation. Amounts used: a) peak II (Fig. 1), 45μg; b) pH 4.6 pellet, 12μg; C) 0-50% $(NH_4)_2SO_4$ fraction of pH 4.6 pellet, 15μg; d) 50-60% $(NH_4)_2SO_4$ fraction of pH 4.6 pellet, 12μg; e) 40-60% $(NH_4)_2SO_4$ fraction of pH 4.6 supernatant, 30μg.

(gel d). The purified TM consistently gave a double band which corresponds to the well known α and β forms of rabbit TM (10-13; cf. Fig. 6). When the Tn fraction was similarly purified, the material obtained at 40-60% $(NH_4)_2SO_4$ saturation showed three protein bands which correspond to the Tn components with no detectable amount of actin and Tm (gel e).

Biological Activity of Embryonic TM-Tn: Column fractions corresponding to peak II (Fig. 1) were fractionated at 0-40% $(NH_4)_2SO_4$ saturation to remove actin. The material was then tested for its ability to confer Ca^{2+}-sensitivity in an in vitro AM ATPase system. A TM-Tn complex prepared by 40-60% $(NH_4)_2SO_4$ fractionation of the dried ether powder extract of adult chicken leg muscles was also simultaneously tested for comparison. As shown in Fig. 4, the embryonic TM-Tn showed a strong inhibition of AM ATPase. Furthermore, over a wide concentration range this activity was comparable to that of adult TM-Tn.

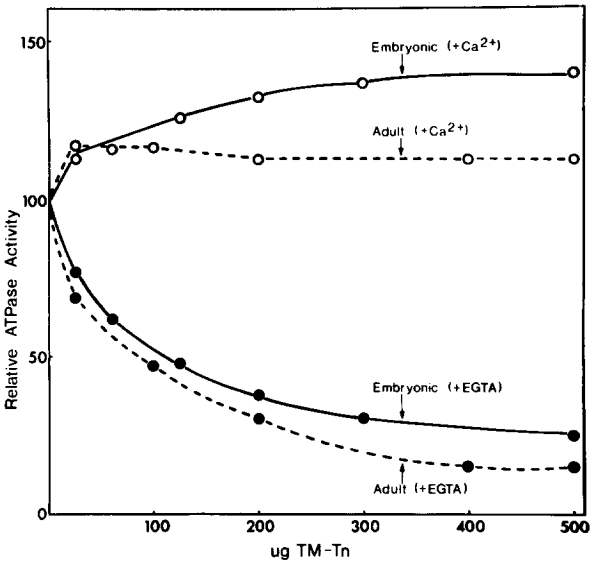

Fig. 4: EGTA inhibition of AM ATPase by TM-Tn isolated from embryonic and adult chicken muscles. For details see Text. Assays were carried out at 25° in a 2 ml incubation mixture containing 25 mM Tris-HCl, pH 7.5, 25 mM KCl, 1 mM $MgCl_2$, 1 mM ATP, 0.62 mg rabbit AM and either 1 mM EGTA or 0.1 mM $CaCl_2$ (7). The activities are expressed in terms of the control AM ATPase in each assay mixture which included the appropriate TM-Tn concentration.

Stoichiometry of the Protein Components of Embryonic TM-Tn Complex: The molecular weights of the components of the purified TM-Tn were first determined by SDS-gel electrophoresis using proteins of known molecular weights (14). The stoichiometry of the components was then estimated from the densitometric scans of gel runs of the complex and the molecular weights of the proteins according to the recent method of Potter (1). A typical gel scan (Fig. 5) indicates that the complex was isolated with a high degree of purity. Using six gel scans of three different preparations, the average value for the stoichiometry of the components TnT:TnI:TnC:TM was calculated as 1.60:1.10:1.00:1.05, respectively. The values for each component, based on TnC as 1.0, ranged as follows: TnT, 1.51-1.70; TnI, 0.95-1.30; and TM, 0.92-1.20. The higher proportion of TnT reported here may be due to the fact that the peaks of TnT and TM were not completely resolved in gel runs and also that the slower moving component of the two bands of TM occasionally comigrates with TnT. Nevertheless, these results indicate that the components of the Ca^{2+}-regulatory complex are not significantly different in chick embryonic and adult muscles.

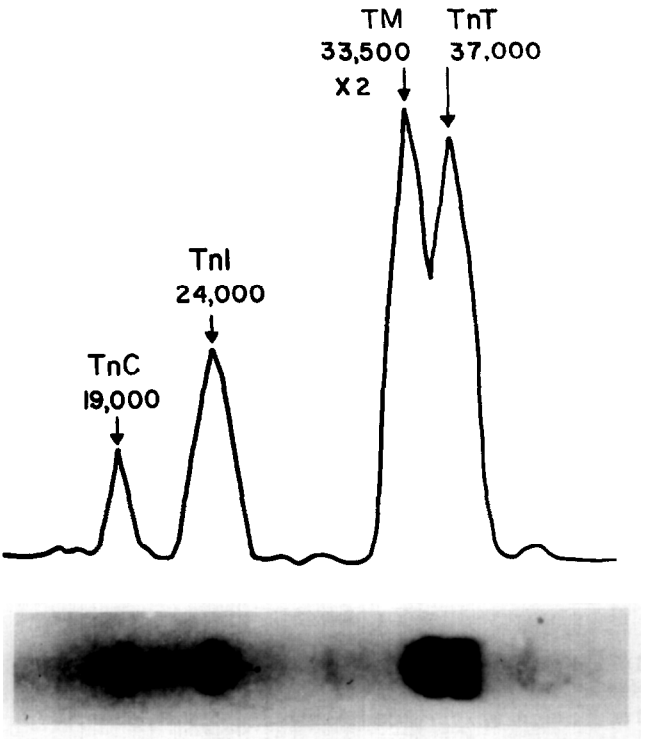

Fig. 5: Densitometric scans of SDS-gel runs of purified embryonic TM-Tn (Fig. 3). For details see Text. Densitometry of gels and stoichiometry of the components of TM-Tn were determined by the method of Potter (1).

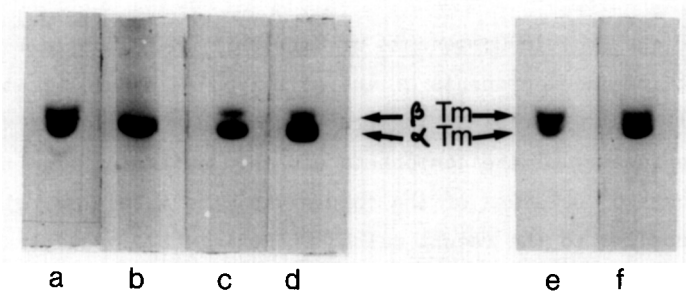

Fig. 6: SDS-gel electrophoresis of carboxymethylated samples of TM purified from embryonic and adult chicken muscles. For details see Text and legends to Figs. 1 and 3. Amounts used: a) embryonic chick breast TM, 12 μg; b) adult chicken breast TM, 10 μg; c) adult rabbit skeletal TM, 12μg; d) mixed sample of rabbit TM (8 μg); e) embryonic chicken leg TM, 10 μg; f) adult chicken leg TM, 12 μg.

Polymorphic Forms of TM in Embryonic and Adult Muscle Fibers: Polymorphic forms of TM containing two kinds of chains, α and β , which differ slightly in amino acid composition and sequence (10-13) are present in both mammalian

and avian striated skeletal muscles (10,15). Slow (red) muscles seem to contain a greater proportion of the β chain than white (fast) muscles (15). We have consistently observed that the embryonic and adult forms of the same muscle fiber contain different polymorphic forms of TM. As shown in Fig. 6, adult chicken breast (fast) muscle consists of only the α subunit (gel b). This is in agreement with the report of Hitchcock et al. (16) but does not support the published report of Cummins and Perry (15) that both subunits are present in TM isolated from adult chicken breast muscle. Rabbit skeletal muscle TM, run as a marker, shows the typical two bands, α and β (gel c) (10-13). When a mixed sample of these two TM samples were run together, the chicken breast TM comigrated with the α form of the rabbit TM (gel d). In contrast, TM isolated from chick embryonic breast muscles contains both α and β subunits (gel a). In the case of chicken leg muscles, which consist of both slow and fast fibers, TM purified from both adult (gel f) and embryonic (gel e) fibers consists of α and β forms. From densitometric scans of the gel runs (not shown here) the α/β ratios listed below were calculated: adult rabbit skeletal, 80:20; adult chicken leg, 50:50; embryonic chick leg, 30:70; and embryonic chick breast, 35:65. This indicates that the β subunit of TM is the prominent species present in embryonic muscles. Furthermore, the two band pattern of the embryonic breast TM is switched to the single α form in the adult stage.

The results presented here describe a simple and highly reproducible method which seems to be ideally suited for the isolation of TM-Tn from small quantities of tissue such as embryonic muscle. This is the first report on the successful isolation and partial characterization of the Ca^{2+}-regulatory complex from embryonic muscles. Using the methodology described here we have recently achieved the synthesis and assembly of the TM-Tn complex from polypeptide chains synthesized in a heterologous cell-free system supplemented with chick embryonic muscle polysomes (17). This observation, together with the results presented here, indicates that Ca^{2+}-regulatory proteins, which are biologically active, are assembled in myofilaments of early embryonic muscles in the same molar ratio as in adult muscles. These results, therefore, do not support the previous conclusion of Hitchcock (4) that the regulatory proteins, possibly Tn, appear only at late stages of muscle development to form a functional contractile apparatus. Our results also indicate that different but closely related structural genes coding for polymorphic forms of TM subunits are expressed in embryonic and adult stages of muscles. Taken together with the published reports on the differences of the heavy and light chains of myosins of embryonic and adult muscles

(18), this supports the concept that there must be a "family of structural genes" coding for various myofibrillar proteins whose expression is regulated during the transition of embryonic to adult stages of muscle fibers.

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