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Actin and Myosin-Linked Calcium Regulation in the Nematode *Caenorhabditis elegans.* Biochemical and Structural Properties of Native Filaments and Purified Proteins†

Harriet E. Harris, Man-Yin W. Tso, and Henry F. Epstein*

ABSTRACT: Calcium regulation of actomyosin activity in the nematode, *Caenorhabditis elegans,* has been studied with purified proteins and crude thin filaments. Actin and tropomyosin have been purified from *C. elegans* and shown to be similar in most respects to actin and tropomyosin from rabbit skeletal muscle. The actin comigrates with rabbit actin on polyacrylamide–sodium dodecyl sulfate gel electrophoresis, forms similar filaments and paracrystals, and activates the Mg^{2+}-ATPase of rabbit myosin heads as efficiently as rabbit actin. Nematode tropomyosin has a greater apparent molecular weight (estimated by mobility on polyacrylamide–sodium dodecyl sulfate gels) than the rabbit protein, yet it forms Mg^{2+}-paracrystals with a slightly shorter periodicity. Native thin filaments extracted from nematodes activate rabbit myosin subfragment 1 Mg^{2+}-ATPase in a calcium sensitive manner; the extent of activation is threefold greater in 0.2 mM $CaCl_2$ than in the absence of calcium. This observation suggests that the thin filaments contain components which are functionally equivalent to vertebrate troponins. Calcium is also required for maximal activation of the Mg^{2+}-ATPase of purified nematode myosin by pure rabbit F-actin. *C. elegans* therefore has both myosin and thin filament-linked calcium regulatory systems. The origin of the actin, tropomyosin, and myosin from different tissues and the use of genetic analysis to answer questions about assembly and function in vivo are discussed.

In muscle, thick and thin filaments are organized in parallel arrays. During contraction, the filaments slide past each other, to give overall shortening of the sarcomeres (Huxley and Niedergerke, 1954; Hanson and Huxley, 1954). The relative movement of the filaments is effected by cross bridges protruding from the thick filaments, which interact with the thin filaments in cycles of attachment and detachment (Huxley, 1969). In all muscles, contraction is regulated by intracellular calcium levels. The site of action of Ca^{2+} may be on either the thick filaments, or the thin filaments, or both, depending on the organism and muscle type (Lehman et al., 1972; Lehman and Szent-Györgyi, 1975). For both thick and thin filament linked regulatory mechanisms, it appears that interactions between cross bridges and thin filaments are blocked at very low calcium concentrations.

It is clear that efficient, controlled contraction is dependent on the structural integrity of a highly complex, multicomponent system. Little is known about the processes regulating the assembly and maintenance of the muscle filaments or their organization into mixed lattices. The use of specific mutants has been extremely helpful in understanding analogous questions in virus assembly (Casjens and King, 1974; Katsura and Kuhl, 1974) and bacterial ribosome functions (Davies and Nomura, 1972). This approach appears feasible in the nematode, *Caenorhabditis elegans,* because paralyzed mutants, in which the body wall muscle cells exhibit disrupted filament lattices, have been found (Epstein and Thomson, 1974; Epstein et al., 1974). There is also a need for a better understanding of the relationship between biochemical reactions in vitro and

† From the Department of Pharmacology, Stanford University School of Medicine, Stanford, California 94305. *Received July 14, 1976.* This work was performed during the tenure of a Science Research Council of Great Britain Fellowship to H.E.H., a McCormick Fellowship through Stanford University School of Medicine to M.-Y.W.T., and a National Genetics Foundation (through the Grant Foundation) and Mellon Foundation Fellowships to H.F.E. The research was supported by grants from the National Institute of Aging (No. AG-00448), the Muscular Dystrophy Associations of America, Inc., the American Heart Association, Inc. through the Santa Clara County Chapter (No. 73-950), and the American Cancer Society through an institutional grant to Stanford University.

the actual physiological mechanisms of force generation and its regulation by calcium. In particular there is at present no satisfactory hypothesis for the relative advantages, if any, of the alternative calcium regulatory mechanisms found in different systems. The present report describes biochemical and structural properties of purified actin and tropomyosin from wild-type nematodes, together with the calcium regulatory mechanisms associated with both myosin and thin filaments. This information, in combination with genetic and other biochemical analyses in *C. elegans* (Waterston et al., 1974; H. E. Harris and H. F. Epstein, 1977, may uncover detailed molecular processes determining myofilament assembly and function in vivo.

Materials and Methods

Nematode Growth and Storage. The wild-type strain, N2, of the nematode *Caenorhabditis elegans* was grown in liquid culture (Sulston and Brenner, 1974) and stored at −20 °C in 50% glycerol.

Purification of Nematode Actin, Myosin, Tropomyosin, and Thin Filaments. All manipulations were performed on ice or at 4 °C. Buffer pH values refer to 4 °C.

Actin; Method A. Nematodes (usually 5–15 g) were washed in 20 mM Tris-HCl[1] (pH 8.1), 50 mM NaCl, 1 mM DTT, 1 mM PMSF, and 1 mM EDTA, suspended in 2 vol ("1 vol" refers throughout to initial wet weight in grams of worms) of the same buffer, and passed twice through a 40-mL French pressure cell at 4000–8000 lb/in.^{2}. The homogenate was washed twice and centrifuged at 8000*g* (8700 rpm, Sorvall SE-12 rotor) for 10 min. The washed pellet was extracted with 1 vol of 20 mM Tris-HCl (pH 8.1), 0.6 M KCl, 5 mM ATP, 5 mM $MgCl_2$, 1 mM DTT, 1 mM PMSF, and 1 mM $CaCl_2$ and centrifuged at 20 000*g* (14 000 rpm, Sorvall SE-12 rotor) for 15 min. The pellet was extracted once more and the combined supernatants clarified by centrifugation at 100 000*g* (40 000 rpm, Beckman type 40 rotor) for 20 min. The supernatant, containing all the major myofilament proteins, was fractionated by gel filtration in the presence of KI and ATP (Stossel and Pollard, 1973; Clarke and Spudich, 1974). A 2.5 × 100 cm Sephadex G-100 column was washed before the sample was applied with 20 mL of 20 mM Tris-HCl (pH 8.1), 0.5 M KI, 5 mM ATP, 5 mM $MgCl_2$, 1 mM DTT, 1 mM PMSF, and 1 mM $CaCl_2$. The sample was made 0.5 M in KI, loaded, and eluted with 10 mM Tris-HCl (pH 8.5), 0.6 M KCl, 0.5 mM ATP, 0.5 mM $MgCl_2$, 1 mM DTT, and 0.1 mM PMSF. Fractions giving a single actin band on 10% sodium dodecyl sulfate–polyacrylamide electrophoresis were pooled, dialyzed against 2 mM Tris-HCl (pH 8.5), 0.2 mM $CaCl_2$, 0.2 mM DTT, and 0.5 mM ATP overnight, and concentrated in an Amicon ultrafiltration cell with a PM 10 membrane (Amicon Corp., Lexington, Mass.). The actin was polymerized by addition of KCl to 50 mM and $MgCl_2$ to 3 mM.

Actin and Myosin; Method B. Worms were washed, homogenized, and washed again as described in method A, except that the low salt buffer was 15 mM sodium phosphate (pH 7.0)–50 mM NaCl–1 mM EDTA–1 mM PMSF–5 mM DTT. Actomyosin and associated proteins were extracted from the washed pellet with high salt. The pellet was suspended in 1 vol of 20 mM Tris-HCl, 0.6 M KCl, 1 mM DTT, and 1 mM PMSF (pH 8.0) and centrifuged at 20 000*g* for 15 min. The extraction was repeated and the combined supernatants centrifuged at 100 000*g* for 3 h. Under these conditions, all the myosin was bound to the actin filaments, which sedimented, while the tropomyosin, dissociated from thin filaments in high salt (Spudich and Watt, 1971), remained in the supernatant, together with paramyosin. Purification of tropomyosin from this 100 000*g* supernatant is described below.

The 100 000*g* pellet was suspended gently in 0.25–0.5 vol of 20 mM Tris-HCl–0.6 M KCl–5 mM ATP–5 mM $MgCl_2$–1 mM DTT–1 mM PMSF (pH 8.0), using a Dounce hand homogenizer, and given a clarifying spin at 100 000*g* for 15 min. The supernatant actin and myosin were separated by gel filtration. A 1 × 120 cm Sepharose 4B column was washed with 20 mL of 20 mM Tris-HCl–0.6 M KI–5 mM ATP–5 mM $MgCl_2$–1 mM DTT–1 mM PMSF (pH 8.0), loaded with sample (made 0.4 M in KI), and washed with a further 20 mL of the KI-containing buffer. Elution was completed with 20 mM Tris-HCl–0.6 M KCl–0.2 mM ATP–1 mM DTT–1 mM PMSF (pH 8.0). Myosin eluted immediately after the void volume, was identified by Ca^{2+}-ATPase activity and polyacrylamide–sodium dodecyl sulfate gel electrophoresis (H. E. Harris and H. F. Epstein, 1977, and was pooled and concentrated by ultrafiltration. Actin-containing fractions were pooled and concentrated and the actin polymerized as described in method A.

Tropomyosin. Tropomyosin and paramyosin were purified from the 100 000*g* supernatant obtained in method B, by a modification of the method of Waterston et al. (1974). The solution was filtered through nylon gauze to remove floating fat, three times its volume of 95% ethanol–2 mM DTT was added with stirring, and the mixture was stirred for 2 h at 25 °C. After centrifugation (20 000*g* for 20 min) the pellet was suspended in ⅓–½ vol (milligrams/gram wet weight worms) of 10 mM potassium phosphate–0.6 M KCl–2 mM DTT (pH 7.6), and dialyzed overnight at 4 °C against this buffer. Soluble proteins (20 000*g* supernatant) were dialyzed exhaustively against 10 mM potassium phosphate–0.1 M KCl–2 mM DTT (pH 6.0). A white precipitate of crude paramyosin was sedimented (20 min at 20 000*g*). Tropomyosin was precipitated from the supernatant as paracrystals by dialysis against 20 mM Tris-HCl (pH 8.3)–50 mM $MgCl_2$, and redissolved when appropriate in 10 mM Tris-HCl (pH 8.0)–50 mM KCl.

Nematode Thin Filaments. Intact thin filaments were extracted by a modification of the method of Szent-Györgyi et al. (1971). A nematode homogenate from about 2 g of worms was washed with low salt buffer as in the method B preparation of actin. The pellet was extracted once with 2 mL of 50 mM KCl, 10 mM potassium phosphate (pH 6.0), 2 mM ATP, 1 mM $MgCl_2$, 1 mM DTT, 1 mM PMSF, and 0.1 mM EDTA, and centrifuged 10 min at 10 000*g*. The supernatant, a clear suspension of thin filaments, was adjusted to pH 7.5. Filaments were used in assays immediately after preparation, as proteolysis is a major problem in crude extracts.

Other Proteins. Rabbit actin was purified from an acetone powder of leg and back muscle by the method of Spudich and Watt (1971). HMM was prepared by limited tryptic digestion of rabbit myosin (Lowey et al., 1969). Rabbit tropomyosin was prepared according to the method of Eisenberg and Kielley (1974) and was a gift of Mr. S. Dilsalver.

Protein Determinations. Protein concentrations were estimated by the microbiuret method (Goa, 1953) or by Lowry's method (Lowry et al., 1951) using bovine serum albumin as a standard.

Gel Electrophoresis. Polyacrylamide–sodium dodecyl sul-

[1] Abbreviations used are: Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; PMSF, phenylmethylsulfonyl fluoride; EDTA, (ethylenedinitrilo)tetraacetic acid; EGTA, [ethylenebis(oxoethylenenitrilo)]tetraacetic acid; DTT, dithiothreitol; HMM, heavy meromyosin; HMM SF-1, heavy meromyosin subfragment 1.

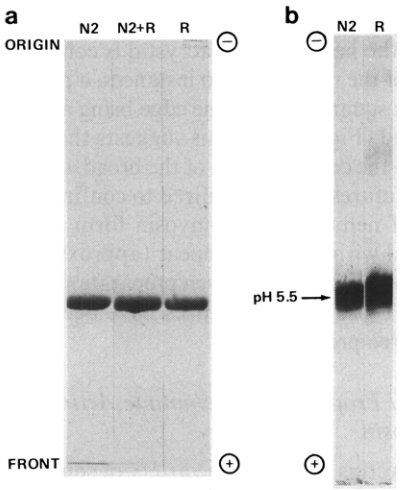

FIGURE 1: (a) Polyacrylamide–sodium dodecyl sulfate (10%) slab gel of 10 μg of nematode actin (N2), 4 μg of nematode actin + 6 μg of rabbit actin (N2 + R), and 6 μg of rabbit actin (R). Nematode actin was prepared by method A. (b) p*I* of nematode actin monomers; pH 3.5–10 gel electrofocusing of 75 μg of nematode actin (N2) (prepared by method A) and 75 μg of rabbit actin (R). Staphylococcal nuclease and bovine pancreatic ribonuclease markers are present in the rabbit actin electrofocusing gel.

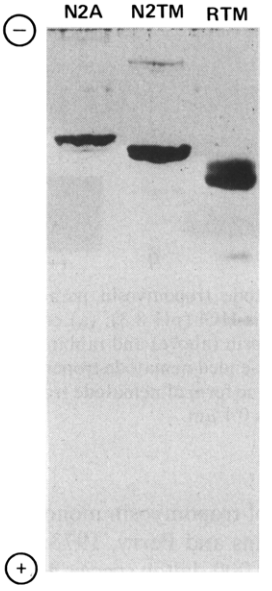

FIGURE 2: Polyacrylamide–sodium dodecyl sulfate (15%) slab gel of nematode actin (N2A, prepared by method B), nematode tropomyosin (N2TM), and rabbit tropomyosin (RTM); 10 μg of each protein.

fate slab gel electrophoresis was performed as described by Epstein et al. (1974). Electrofocusing gels (0.6 × 11 cm) contained 10% acrylamide monomer (Eastman Organic Chemicals, Rochester, N.Y.), 0.267% *N,N*-methylenebisacrylamide (Bio-Rad, Richmond, Calif.), 8 M urea, 1% (v/v) pH 3.5–10 ampholines (L.K.B.), 0.03% (v/v) *N,N,N′,N′*-tetramethylenediamine, and 5 ppm (w/v) of riboflavin. Proteins were heated for 30 min at 60 °C in 8 M urea, 0.1% (v/v) β-mercaptoethanol, 0.01 M sodium phosphate (pH 7.0), and 60% (w/v) sucrose, applied to the gels, and layered with 8 M urea–1% ampholine. Electrode contacts were made with 2% (v/v) ethanolamine (cathode) and 1.25% phosphoric acid (anode). Tubes were run at 200 V for 16 h. Gels were immersed in 10% trichloroacetic acid for 30 min to remove ampholines, before staining in 0.25% (w/v) Coomassie brilliant blue. The pH gradient of an unstained gel was determined by soaking 0.5-cm sections in 2 mL of water for 1 h and measuring the pH values.

ATPase Assay. Myosin ATPase was measured as the rate of release of radioactive phosphate from [γ-^{32}P]ATP. The procedure is based on that of Spudich (1972). Incubation mixtures contained 25 mM Tris-HCl (pH 8.1), 2.5 mM $MgCl_2$, 0.5 mM EGTA, or 0.2 mM $CaCl_2$ in 0.1–0.2 mL of [γ-^{32}P]ATP (New England Nuclear or prepared in this laboratory), 0.01–0.03 μCi was added to 2.0 mM at zero time, and the reaction was allowed to proceed for 15 min. Rates are linear over this period. Reactions were stopped with 0.4 mL of 0.5 N perchloric acid–1 mM KH_2PO_4. Inorganic phosphate was precipitated by 0.5 mL of 5% ammonium molybdate–33 mM triethylamine hydrochloride (pH 5.0). After 15–30 min, the precipitates were collected on glass fiber filters (Whatman GF/C, 2.4 cm) and washed with 5 2-mL volumes of cold 0.01 M triethylamine (pH 5.0), 0.03 M ammonium molybdate, 0.1 mM KH_2PO_4, and 0.2 N perchloric acid. The dried filters were counted in toluene (10 mL) using a Nuclear-Chicago Isocap/300 or Packard Model 3900 scintillation counter. Solution phosphate activity and precipitated counts are related by a proportionality constant which is standard for a given set of counting conditions.

Electron Microscopy. Samples on Formvar film, carbon-coated grids were washed with 0.1 M KCl (actin) or 20 mM Tris-HCl–50 mM $MgCl_2$ (tropomyosin) and negatively stained with 1% (w/v) uranyl acetate. Micrographs were taken with a Siemens Elmiskop Ia microscope, operating at 60 kV. Mg^{2+}-tactoids of rabbit skeletal muscle tropomyosin with a periodicity of 39.5 nm (Caspar et al., 1969) were used for calibration.

Results

Polypeptide Composition of Purified Nematode Actin and Tropomyosin

Nematode actin obtained by method A migrates as a single species on polyacrylamide–sodium dodecyl sulfate gels with an identical mobility to rabbit skeletal muscle actin (Figure 1a). The comigration of these two proteins suggests that they have the same monomer molecular weight of 42 000 (Elzinga et al., 1973). The two actins migrate on heavily loaded electrofocusing gels, with the same apparent pI of 5.5 (Figure 1b).

The alternative purification method (B) has the advantage that myosin, paramyosin, and tropomyosin may also be obtained rapidly from the same batch of nematodes. Yields are higher when method B is used, but the actin contains more trace contaminants. A typical method B preparation yielded 2.5 mg of actin of 90% purity (estimated by densitometry of a Coomassie blue stained gel) from 10 g of nematodes, and about 0.4 mg of tropomyosin.

Paramyosin is the only significant contaminant of nematode tropomyosin, although preparations free of it may be obtained. Nematode tropomyosin has an electrophoretic mobility on polyacrylamide–sodium dodecyl sulfate gels between rabbit skeletal muscle actin and tropomyosin (Figure 2). These three proteins migrate in the same relative positions in the sodium dodecyl sulfate–phosphate buffer system of Weber and Osborn (1969). Using M_r values of 37 000 and 35 000 for the two rabbit tropomyosin polypeptides, respectively (Eisenberg and Kielley, 1974), and 42 000 for actin, nematode tropomyosin has an M_r of 40 000. There is no evidence for two closely mi-

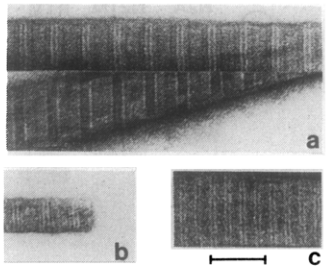

FIGURE 3: Nematode tropomyosin paracrystals formed in 50 mM $MgCl_2$–20 mM Tris-HCl (pH 8.3): (a) comparison of periodicities of nematode tropomyosin (above) and rabbit skeletal muscle tropomyosin (below); (b) square-ended nematode tropomyosin paracrystal; (c) alternative paracrystalline form of nematode tropomyosin, with shorter periodicity. Scale bar is 0.1 μm.

grating classes of tropomyosin monomer as in rabbit skeletal muscle (Cummins and Perry, 1973; Eisenberg and Kielley, 1974) or for a 30 000-dalton species as in vertebrate nonmuscle tissues (Cohen and Cohen, 1972; Fine and Blitz, 1975).

Purified actin and tropomyosin from *C. elegans* were free of significant protein components having molecular weights between 15 000 and 60 000. The size of calcium regulatory proteins associated with muscle thin filaments has generally been in this range (Lehman et al., 1972).

Details of nematode myosin purification and properties will be described elsewhere (H. E. Harris and H. F. Epstein, 1977.

Assembly of Nematode Actin and Tropomyosin into Ordered Structures

In muscle thin filaments, actin monomers are organized in polar strands, which form a double helix of 36–37-nm pitch (Hanson, 1968). Tropomyosin is located along the length of these filaments (Ebashi and Endo, 1968) in the deep groove of this helix (O'Brien et al., 1971). Because these proteins have very specific intermolecular contact sites, purified actin and tropomyosin can assemble in vitro into organized structures. The ability to form such structures indicates retention of native conformation during purification. Repolymerized nematode actin consists of long filaments that are 6–7 nm in diameter. Polarity in these filaments has been demonstrated by the formation of a unidirectional "arrowhead" pattern upon the addition of rabbit HMM or HMM SF-1 as described for rabbit actin by Huxley (1963). Paracrystalline arrays of nematode F-actin form in 50 mM $MgCl_2$ which closely resemble rabbit skeletal muscle actin Mg^{2+}-paracrystals (Hanson, 1968; Spudich et al., 1972), and which exhibit a periodicity of 36–37 nm, corresponding to the helical repeat.

Rabbit muscle tropomyosin forms dihedral paracrystals with a characteristic 39.5-nm repeat in 50 mM $MgCl_2$–20 mM Tris-HCl (pH 8.3) (Caspar et al., 1969). These structures result from polar, head-to-tail associations of tropomyosin molecules which are stacked antiparallel to neighboring rows. The 39.5-nm repeat represents a minimum value for the molecular length. Purified nematode tropomyosin also forms dihedral paracrystals in 50 mM $MgCl_2$, which resemble those of the rabbit protein in having a characteristic double, light band at approximately 39-nm intervals. In detail, however, the paracrystals clearly differ. The light doublet is narrower and less prominent for the nematode protein, while the overall repeat is shorter (38.2 ± 0.2 nm). Figure 3a shows a direct comparison of nematode and rabbit skeletal muscle tropomyosins; the micrographs were taken in sequence without any alteration of the electron microscope settings. The periodicity of the nematode paracrystal is consistently 3–4% less than that of the rabbit. In two instances, a paracrystal has been seen with a square-cut end, the edge being about 20 nm beyond a light band (Figure 3b). This suggests that all the molecules may end in the central region of the broad, dark band, but more detailed pictures will be required to confirm this. Some preparations of nematode tropomyosin form a different type of paracrystal with a shorter repeat (approximately 20 nm, uncalibrated) (Figure 3c). A given preparation yields only a single structural type. Both structures have been observed in paramyosin-free preparations.

Functional Properties of Nematode Actin and Tropomyosin

Actin. Actins from a wide variety of sources are capable of activating the Mg^{2+}-ATPase of myosin or of its active proteolytic subfragments, HMM and HMM SF-1. This property is thought to be biochemically analogous to the interaction of actin and myosin during contraction. Nematode actin activation of rabbit skeletal muscle HMM was tested in 25 mM Tris-HCl (pH 8.1)–2.5 mM $MgCl_2$, at 30 °C. Reaction mixtures contained 0.08 mg mL^{-1} HMM. In 0.2 mM $CaCl_2$, actin (both nematode and rabbit) at 0.1 mg mL^{-1} increased the ATPase from 0.082 μmol min^{-1} (mg of HMM)$^{-1}$ to 0.88 μmol min^{-1} (mg of HMM)$^{-1}$. In the absence of calcium (0.5 mM EGTA) corresponding activities, in μmol min^{-1} (mg of HMM)$^{-1}$ were 0.093 (HMM alone), 0.88 (HMM + nematode actin), and 0.90 (HMM + rabbit actin). Clearly the nematode actin activates HMM as efficiently as rabbit and this activation is unaffected by calcium.

Tropomyosin. Tropomyosin is expected to bind to F-actin under conditions where native thin filaments remain intact. However, the two purified proteins should not associate in high salt at alkaline pH, a condition which releases tropomyosin from thin filaments (Spudich and Watt, 1971). We compared the ability of nematode tropomyosin to bind to rabbit F-actin at low and high ionic strengths. Mixtures of 140 μg of rabbit actin and 30 μg of nematode tropomyosin in 0.25 mL of 5 mM Tris-HCl (pH 8.0) containing either 0.05 or 0.67 M KCl were incubated on ice for 30 min. The solutions were centrifuged at 100 000*g* for 3 h to pellet the F-actin. The amounts of tropomyosin remaining in the supernatants were compared by densitometry of 10% polyacrylamide–sodium dodecyl sulfate slab gels, stained with Coomassie brilliant blue. At 0.05 and 0.67 M KCl, supernatant tropomyosin was 4.5 and 19.0, respectively (in arbitrary density units), compared with 21.0 for a control containing no actin. Therefore, nematode tropomyosin can bind to rabbit F-actin at low salt, but dissociates from it at high salt.

Calcium Regulation of Nematode Native Thin Filaments

In many muscles, thin filament components are responsible for calcium regulation of contraction. Purified F-actin and tropomyosin are necessary but insufficient for reconstitution of calcium-sensitive filaments; the troponin complex is required for regulation. Native thin filaments, extracted from homogenates of *C. elegans* as described under Materials and Methods, contain actin monomers, tropomyosin monomers, and several smaller polypeptides as the principal components, apart from a myosin contaminant (Figure 4). Electron microscopy of negatively stained samples of these preparations confirms that they contain intact thin filaments resembling F-actin in appearance.

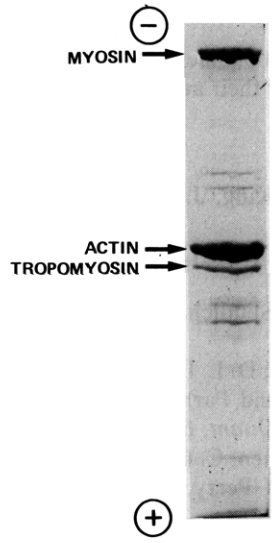

FIGURE 4: Polypeptide composition of nematode thin filament preparation, 25 μg of protein on a 10% polyacrylamide–sodium dodecyl sulfate gel.

These filaments, unlike those of purified actin, activate myosin ATPase in a Ca^{2+}-sensitive manner. The activated Mg^{2+}-ATPase of a rabbit HMM SF-1 and nematode thin filament mixture was increased over threefold by the addition of Ca^{2+} (Table I). Although no purification has yet been attempted, these results suggest that some of the thin filament minor components have troponin-like activity.

Calcium Regulation of Nematode Myosin

A difficulty in studying *C. elegans* myosin is that, since the starting material for all purifications is necessarily a homogenate of whole animals, rather than dissected muscle tissue, all crude extracts have considerable proteolytic activity. In addition, high salt extracts from homogenized nematodes have a higher proportion of contaminating proteins and lower concentration of myosin than would actomyosin extracts from muscle, while the myosin itself is only weakly activated by actin. For these reasons, attempts to determine whether the myosin is calcium sensitive in crude extracts, by the competitive actin-binding assay of Lehman and Szent-Györgyi (1975), were inconclusive. We therefore used column purified myosin, which is more stable, for most of our assays.

The nematode myosin obtained by Sepharose 4B chromatography is over 90% pure, and contains a 210 000-dalton heavy chain and two classes of light chain (16 000 and 18 000 daltons) (H. E. Harris and H. F. Epstein, 1977. The ATPase of the myosin alone in 2.5 mM $MgCl_2$, with or without calcium, is low, and comparable to that of rabbit skeletal muscle myosin, under the same conditions (Table II). Purified rabbit actin stimulates nematode myosin Mg^{2+}-ATPase approximately twofold at low (0.2 mM) calcium concentrations. Although this effect is very small compared with the 15- to 20-fold stimulation of rabbit myosin under identical conditions, it is reproducible, increases with actin concentration, and has been demonstrated with four separate myosin preparations (H. E. Harris and H. F. Epstein, 1977. In the presence of EGTA, i.e. with calcium effectively removed, actin activation of nematode myosin is strongly inhibited. The extent of EGTA inhibition varies from 50 to 10 with different preparations of myosin. That the calcium sensitivity must be caused by a direct effect of calcium on the nematode myosin is clearly demonstrated by the absence of calcium sensitivity in the parallel experiments with rabbit myosin (Table II).

TABLE I: Calcium Regulation by Native Thin Filaments.[a]

Proteins Assayed	Mg^{2+}-ATPase Ca^{2+}	Mg^{2+}-ATPase EGTA
HMM SF-1, 10 μg	2.5	2.5
Native thin filaments, 90 μg	25.5	18.2
HMM SF-1, 10 μg, + native thin filaments, 90 μg	48.4	26.9
Calcd thin filament activation of HMM SF-1	20.4	6.2

[a] Reactions were performed at 25 °C in 2.5 mM Tris-HCl (pH 8.1)–2.5 mM $MgCl_2$ in a total volume of 0.1 mL. Each reaction was initiated by the addition of ATP to 2.0 mM and stopped after 15 min. Activities are in (μmol min^{-1}) × 10^4. In appropriate experiments, $CaCl_2$ was 0.2 mM and EGTA was 0.5 mM.

TABLE II: Calcium Regulation of Nematode Myosin.[a]

Reaction Components	Sp Act.[b] (μmol min^{-1} (mg of myosin)$^{-1}$) Nematode	Rabbit
Myosin, 15 μg, + $CaCl_2$, 0.2 mM	0.015[c]	0.014 ± 0.006
Myosin, 15 μg, $CaCl_2$, 0.2 mM, + rabbit actin, 20 μg	0.036 ± 0.001	0.24 ± 0.03
Myosin, 15 μg, + EGTA, 0.5 mM	0.018 ± 0.001	0.02 ± 0.01
Myosin, 15 μg, EGTA, 0.5 mM, + rabbit actin, 20 μg	0.014 ± 0.001	0.27[c]

[a] Comparison with rabbit myosin under identical conditions. Assay mixtures contained 60 mM KCl, 25 mM Tris-HCl (pH 8.1), and 2.5 mM $MgCl_2$; other components as tabulated; reaction temperature, 25 °C. [b] Standard deviations for two determinations. [c] Single determination.

Discussion

As part of our study of the myofilament proteins of *C. elegans*, we have investigated the calcium control of the actomyosin ATPase, and find that the organism has both thin filament and myosin-linked regulatory systems. Two thin filament components, actin and tropomyosin, have been isolated and characterized.

Purified nematode actin forms filaments with very similar structural and functional properties to rabbit skeletal muscle F-actin. Consistent with these findings is the apparent similarity of molecular size and charge of nematode and rabbit actin monomers.

Nematode tropomyosin Mg^{2+}-paracrystals grossly resemble those of rabbit skeletal muscle tropomyosin, having a bipolar repeating pattern, with a distinctive pair of light bands at about 38–40-nm intervals. The exact periodicity is slightly less for nematode than for rabbit tropomyosin, but much closer to this and other muscle tropomyosins than to the 34.5-nm repeat of nonmuscle tropomyosins (Cohen and Cohen, 1972; Fine and Blitz, 1975). A detailed comparison of the paracrystals from rabbit and nematode indicates that the molecular packing, relative to the banding pattern, is not the same in both cases. In the rabbit paracrystals, the ends of the molecules are thought to be in the white bands (McLachlan and Stewart,

1975). The double, light bands are less well defined in the nematode paracrystals, and additional evidence leads to the tentative conclusion that the molecular ends are not located in the light bands, but in the broad, dark bands. The band pattern and periodicity of nematode tropomyosin resemble those of another invertebrate muscle tropomyosin (lobster striated muscle tropomyosin) (Regenstein and Szent-Györgyi, 1975) more than those of vertebrate muscle tropomyosins.

The mobility of nematode tropomyosin on sodium dodecyl sulfate-polyacrylamide gels is slow, compared with rabbit skeletal muscle tropomyosin. This may reflect an anomalous mobility on electrophoresis, as noted for other tropomyosins (Regenstein and Szent-Györgyi, 1975). Alternatively, it might indicate a true molecular weight difference. The value for minimum molecular length given by the paracrystal periodicity (Cohen et al., 1972) does not help to establish the relative molecular weights of rabbit and nematode tropomyosins, as there could be different degrees of end overlap (McLachlan and Stewart, 1975) in the two types of paracrystalline array.

A problem in assessing the biological implications of the biochemical properties of nematode proteins is that, since proteins are purified from a homogenate of whole animals, the cellular origin of the isolated components has not been demonstrated directly. For example, we have no evidence regarding the original location of the purified actin, although it is likely to have come from both muscle and nonmuscle cells. We believe that it is probable, though not proved, that the nematode has dual calcium regulatory systems, within the same muscles, for the following reasons. Body wall muscle is the predominant muscle type in *C. elegans*, and the only muscle capable of contributing biochemically significant quantities of myofilament proteins to our preparations. This muscle accounts for a substantial proportion of total myosin (Epstein et al., 1974). Furthermore, no nonmuscle myosin has yet been shown to be calcium regulated (Lehman, 1976). Tropomyosin is found in all muscles, irrespective of mode of regulation. Since it purifies as a single molecular weight species from *C. elegans*, is larger than known cytoplasmic tropomyosins (30 000 daltons; Fine and Blitz, 1975), and is a major component, with actin and myosin, of thin filament preparations, it seems likely to be a component of a muscle, thin-filament calcium regulatory system.

The studies described in this paper suggest that it may be possible to use a combination of biochemical and genetic techniques with *C. elegans* to answer several important questions in muscle physiology and development. At present, we do not understand the significance of the location of calcium regulatory proteins. Components responsible for this function may be associated with either thick or thin filaments, or both. The last alternative appears to be the case for *C. elegans* muscle and has been shown in the larger nematode, *Ascaris lumbricoides* (Lehman and Szent-Györgyi, 1975; Lehman, 1976), as well as many other invertebrate phyla (Lehman et al., 1972, 1974). Mutant nematodes found defective in either or both regulatory systems might prove helpful to our understanding of this phenomenon. Another problem which may prove susceptible to genetic analysis is the specification of uniform thin filament length during sarcomere formation. Pure actin in vitro assembles into filaments heterogeneous in length. The genetic approach has been successful for the analogous problem of length determination in λ phage tail assembly (Katsura and Kuhl, 1974). Work is in progress on screening paralyzed mutants of *C. elegans* for defects which may illuminate these areas of interest.

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The Progeny of Rabbit Articular Chondrocytes Synthesize Collagen Types I and III and Type I Trimer, but Not Type II. Verifications by Cyanogen Bromide Peptide Analysis†

Paul D. Benya,* Silvia R. Padilla, and Marcel E. Nimni

ABSTRACT: The radioactive collagens synthesized by the fourth subculture progeny of rabbit articular chondrocytes were extracted and purified after limited pepsin digestion by neutral and acid salt precipitation. In order to identify the different types of collagen present, denatured collagen chains were fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis on 5% gels, electrophoretically eluted, and cleaved with cyanogen bromide, and the resultant peptides were fractionated by a new sodium dodecyl sulfate electrophoresis system (tris(hydroxymethyl)aminomethane-borate buffer, 15% gels). Comparison of these separate peptide profiles with those from α1(I) and α1(III) collagen chains permitted the unambiguous identification of these chains in the radioactive collagen synthesized by chondrocytes. Although cartilage slices predominantly synthesized α1(II) chains, only α1(I) chains were made by cells in fourth subculture. A large fraction of these α1(I) chains could not be accounted for by the presence of type I collagen. While in a native, triple-helical conformation, some of these extra α1(I) chains were completely separated from type I collagen by their solubility at pH 8.0 in 2.6 M NaCl and therefore identified as $[\alpha 1(I)]_3$, type I trimer. In addition to type I collagen and type I trimer, these chondrocyte progeny also synthesized type III collagen and two new collagen chains, X and Y. Each collagen type was further characterized by carboxymethylcellulose chromatography and its distribution between the medium and the cell layer. These findings support the idea that cultured chondrocytes assume a collagen phenotype similar to that of their undifferentiated mesenchymal cell precursors.

The chondrocyte phenotype has been shown to be unstable under conditions of in vitro growth. Variation in the culture medium, high plating densities, the presence of embryo extract, and the presence of fibroblasts caused loss of chondrocyte polygonal morphology, elimination of metachromatic matrix formation, and decreased incorporation of radioactive sulfate, while increasing cell mobility and proliferation (Coon, 1966; Abbott and Holtzer, 1968; Bryan, 1968; Marzullo and Lash, 1970). After cartilage collagen, $[\alpha 1(II)]_3$ or type II collagen, was identified as a specific gene product restricted to cartilagenous tissues (Miller and Matukas, 1969; Miller, 1971a, 1973; Miller et al., 1971; Strawich and Nimni, 1971), chondrocyte cultures were analyzed to determine their collagen phenotype. Layman, et al. (1972) demonstrated the synthesis of α2 chains by rabbit articular chondrocytes in monolayer culture indicating the presence of type I collagen, $[\alpha 1(I)]_2\alpha 2$. A similar observation was made by Schiltz et al. (1973) for chick chondrocytes cultured in the presence of embryo extract or 5-bromo-2′-deoxyuridine. Reexamination of the chick chondrocyte collagen phenotype, with the added sophistication of cyanogen bromide peptide analysis of isolated collagen α chains, provided the additional information that cloned senescent chondrocytes or those grown in 5-bromo-2′-deoxyuridine cease making type II cartilage collagen and synthesize both type I collagen and $[\alpha 1(I)]_3$, type I trimer (Mayne et al., 1975, 1976).

Recently we have presented data (Cheung et al., 1976) suggesting that rabbit articular chondrocytes in primary culture produce type III collagen and a new hydroxyproline-containing collagen chain, X, in addition to type II collagen. This analysis was performed by $NaDodSO_4$[1] polyacrylamide gel electrophoresis ($NaDodSO_4$ electrophoresis) on 5% gels (permitting the resolution of these additional collagens) rather than by conventional carboxymethyl (CM)-cellulose chromatography. Subsequent subcultures produced type I collagen, an elevated $\alpha 1{:}\alpha 2$ ratio, and an increased proportion of X and type III collagen.

In the present communication a new $NaDodSO_4$ electrophoresis system for analysis of collagen cyanogen bromide peptides is presented and standardized. This technique allowed the individual collagen peaks resolved by $NaDodSO_4$ electrophoresis on 5% gels to be unambiguously identified. Coupled with analysis by CM-cellulose chromatography and differential salt precipitation, these data demonstrated that the progeny of rabbit articular chondrocytes in their fourth subculture do not make type II collagen, but synthesize type I

† From the Rheumatic Disease Section, Departments of Medicine and Biochemistry, School of Medicine, University of Southern California, Los Angeles, California 90033. *Received August 4, 1976.* This research was supported by grants (DE 02848, DE 00094, AM 00094, AM 10358, and AM 16404) from the National Institutes of Health, Bethesda, Maryland 20014.

[1] Abbreviations used: $NaDodSO_4$ electrophoresis, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; CM-cellulose, carboxymethylcellulose; DMEM, Dulbecco's modified Eagle medium; Tris, tris(hydroxymethyl)aminomethane.