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STRUCTURE AND FUNCTION OF MYOSIN OF THE NEMATODE *Caenorhabditis elegans*

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Abbreviations

HMM	heavy meromyosin
LMM	light meromyosin
S-1	subfragment-1 of myosin
S-2	subfragment-2 of myosin
A	monomer in F-actin
M	myosin head
NTM	native tropomyosin
TM	tropomyosin
TN	troponin
PEP	phosphoenolpyruvate
PK	pyruvate kinase
DTT	dithiothreitol
SDS	sodium dodecyl sulfate
PAGE	polyacrylamide gel electrophoresis
TCA	trichloroacetic acid
PCA	perchloric acid
PMSF	phenylmethylsulfonyl fluoride
TLCK	tosyl-L-lysine chloromethylketone
EGTA	ethyleneglycol bis-(β-aminoethylether)-N,N,N',N7,-tetraacetate
TNBS	2,4,6-trinitrobenzenesulfonate
TNP	trinitrophenyl

GENERAL INTRODUCTION

Outline of Muscle Research

Muscle contraction is one of the fundamental processes in animal life. The basic unit of muscle structure and function is the sarcomere which contains a single lattice of interdigitating thick and thin filaments. The sarcomeres are bounded on opposite ends by the Z-lines. A.F. Huxley and Niedergerke (1) and H.E. Huxley and Hanson (2) simultaneously and independently proposed a theory that has become known as the sliding-filament model for muscle contraction. It is now generally accepted that changes in the length of the sarcomere are caused by longitudinal sliding of two sets of filaments (thick and thin filaments) relative to each other, without changes in the length of the filaments themselves (Fig. 1).

Myosin, the major component of thick filaments, has a molecular weight of approximately 4.8×10^5. When denatured, it yields two heavy chains of about 200,000 daltons each and four light chains of about 20,000 daltons each. The native molecule consists of rod segment composed of the C-terminal regions of two heavy chains interwinded in parallel -helical structure, and two globular heads each composed of the N-terminus of one heavy chain and two light chains (3-6)(Fig. 2).

Interactions between the rod segments of the myosin molecules are necessary for the assembly of the thick filament. The globular heads of the myosin molecules, which contain the myosin-associated ATPase activity and the actin-binding sites, project from the thick filaments in a helical arrangement to form the cross-bridges responsible for the interaction of the thick

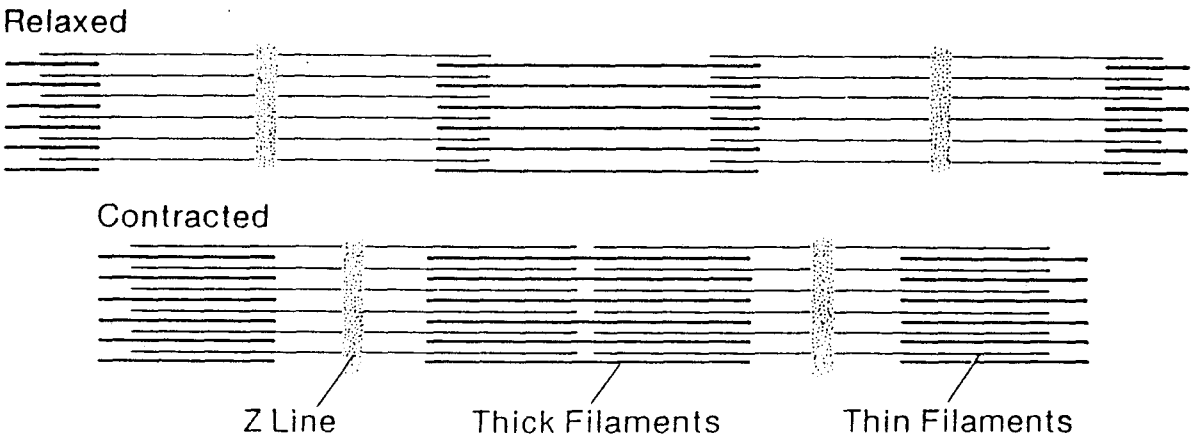

Fig. 1 Sliding filament model

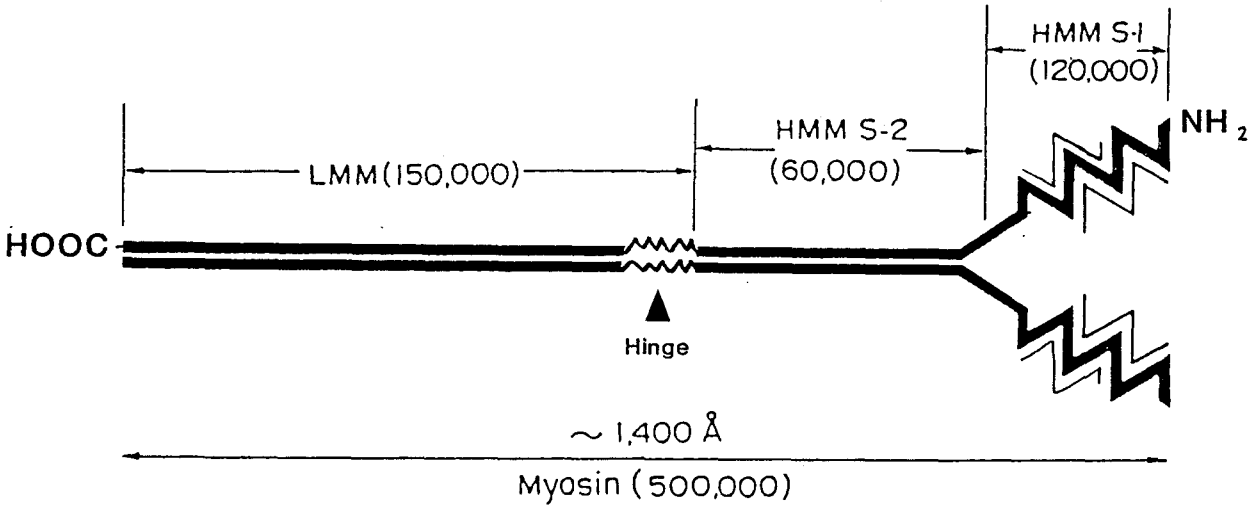

Fig. 2 Structure of myosin molecule

and thin filaments (7). The significance of the myosin light chains associated with the heads is still obscure, although in molluscan muscle they are believed to play a role in calcium regulation (8), and in smooth muscle the contraction is regulated by the phosphorylation of light chain by Ca-dependent light chain kinase and its dephosphorylation by phosphatase.

Thick filaments in a variety of invertebrates, including molluscs, annelids, and some arthropod classes, contain an additional protein, paramyosin. Native paramyosin is composed of two -helical polypeptides that form a rod like molecule. Paramyosin is located in the core of the thick filament (9).

Actin is the main constituent of the thin filaments. Actin monomer, G-actin, has a molecular weight of about 42,000. In thin filaments, the monomers are polymerized in polar strands which wind around each other to form double-helical F-actin.

In vertebrate skeletal muscle and other muscle types which exhibit actin-linked calcium regulation, two additional proteins, tropomyosin and troponin, are associated with actin in the thin filaments (10)(Fig. 3). Tropomyosin, a rod-shaped molecule, is thought to be situated in the grooves between two actin strands, effectively covering seven actin monomers of one strand. The native tropomyosin molecule is a helical two-stranded coil that is composed of two very similar or identical polypeptides.

Troponin is a protein complex (74,000-76,000 daltons) that works in conjunction with tropomyosin to regulate contractile activity in vertebrate skeletal muscle. Troponin consists of three subunits: TnC containing the calcium-binding site, TnI, the

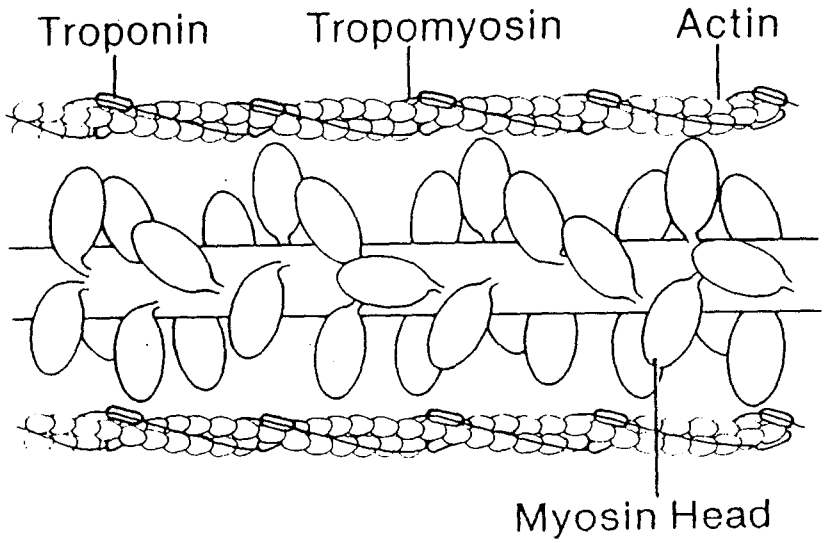

Fig. 3 Interdigitation of a thick filament with neigboring thin filaments.

inhibitory component, and TnT, the tropomyosin-binding component (11). Muscles which exhibit myosin-linked, rather than actin-linked, calcium regulation contain little or no troponin. However, in these muscles tropomyosin is still associated with the thin filament.

Since the myosin cross-bridges possess ATPase activity, and since this activity is enhanced by the presence of actin, it appeared to be very likely that these heads must be intimately involved in the machinery for converting chemical energy into mechanical work. But the mechanism involved is only poorly understood and is the subject of current work by many muscle biophysicists. However, a number of working models have been suggested of which the most basic is probably that of H.E. Huxley (12). He suggested that muscle contraction occurs by a cross-bridge cycle in which a cross-bridge would first attach to an actin filament in a particular orientation. The release of chemical energy that accompanies the release of the products ADP and Pi would then result in a change in orientation of the actin-attached cross-bridge, and this would result directly in the relative sliding of actin and myosin filaments. The cross-bridge would then detach from the actin filament and prepare itself for another attachment-detachment cycle (Fig. 4).

The central problem in a muscle research is to establish precisely how the cross-bridge generates force while it is attached actin. Current theories predict that force is produced either by an angular movement of the entire myosin head (13,14)

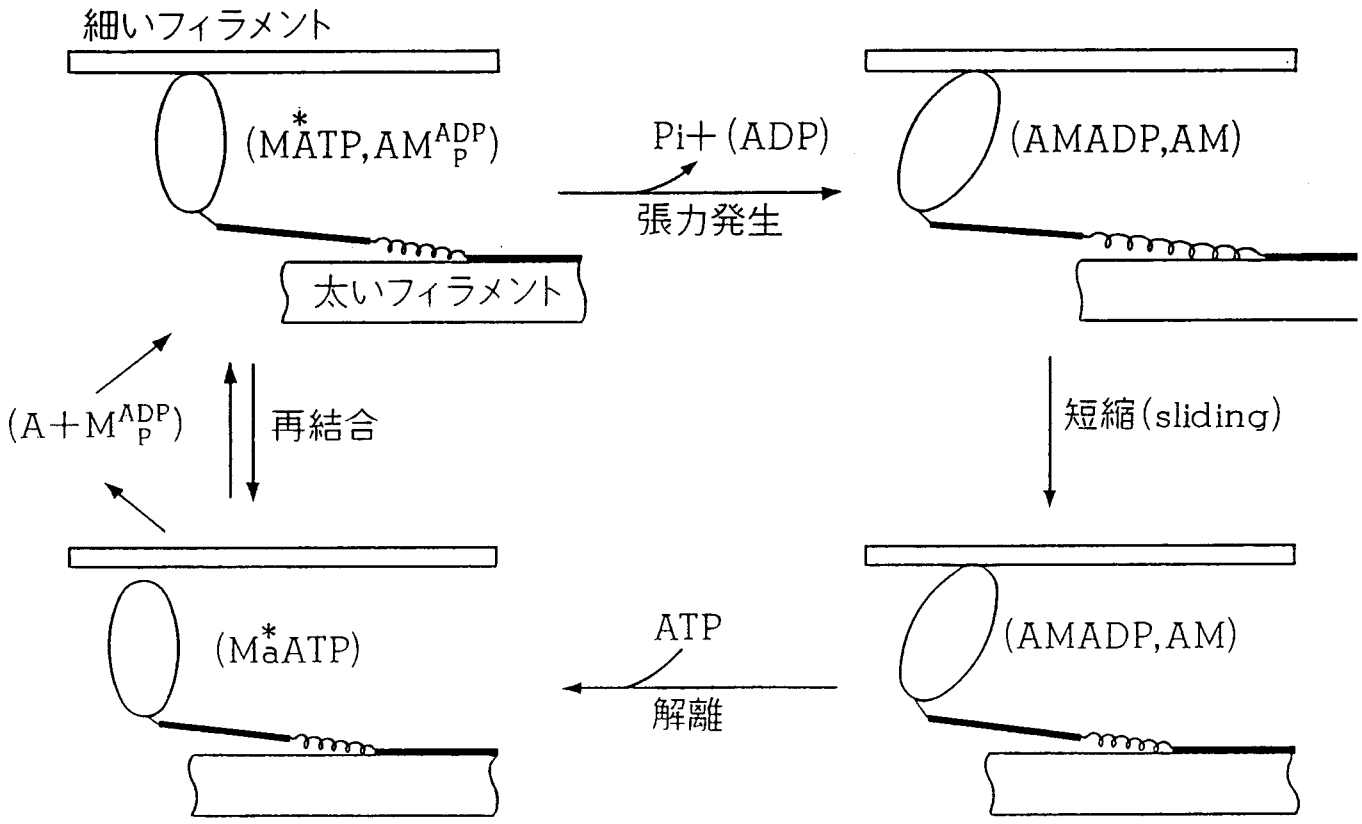

Fig. 4 Mechanism of muscle contraction

(helix-coil) transition within the part of rod may produce force (15).

For understanding the precise mechanism of actin-myosin interaction and the exact location and structure of actin-binding and ATP-binding sites on the myosin molecule it is required to know the primary and tertiary structure of myosin, particularly of primary structure of myosin heavy chains (MHCs). However, like most other contractile proteins, sarcomeric MHCs exist as different isoforms, and it appears that there are two different MHCs within the same muscle type (17,18). These findings do not provide clear interpretations from biochemical and biophysical experiments. Therefore, a new system which contains a single form of MHC is required for further investigation of muscle contraction.

Present work demonstrates that the nematode Caenorhabditis elegans is very useful for muscle research, especially structure and function of myosin molecule which play a key role in muscle contraction.

Nematode as Research Animal

Caenorhabditis elegans, a free-living soil nematode, was first proposed as a model for genetic studies by E. Dougherty and co-workers (19). The pioneering work of S. Brenner (20) on the genetics firmly established C.elegans as a model for higher animals. The classic studies established that nematode are very favorable for developmental studies.

The adult hermaphrodite of C.elegans is about 1 mm in length

and contains about 800 somatic cells (21). It has a simple morphology typical of a rhabditoid nematode. C.elegans is easily and inexpensively grown under controllable laboratory conditions. Normally, it is kept at 20°C on agar plates with E.coli as food source (20). The life cycle of C.elegans is about 3.5 days at 20°C.

C.elegans has several distinct muscle types: body wall, pharynx, vulva, anal sphincter, intestinal and uterine muscles. The body wall musculature is by far the predominant in terms of cell number and mass, and therefore has been the subject of the most intensive analysis. In the adult, there are 95 spindle-shaped, interlocking body wall muscle cells, arranged in four longitudinal single-layered bundles (two dorsal and two ventral) just under the hypodermis. As with other nematodes, the body wall muscle of C.elegans is "obliquelly striated". That is, thick and thin filaments are still organized into the I, A, and H bands typical of vertebrate striated muscle. However, the filaments are at a 6° angle to the dense body rows, not 90° as in vertebrate striated muscle, though the filaments, but not the sarcomeres that contain them, are parallel to the long axis of the muscle cell (Fig. 5).

Mutants affecting the characteristic pattern of motility of C.elegans can be easily identified, and microscopic examination of these "uncoordinated", or unc strains, in the living animal by polarized light microscopy, or more carefully, by electron microscopy has led to the identification of 22 genes that produce altered muscle phenotypes (22), and also 6 genes identified as

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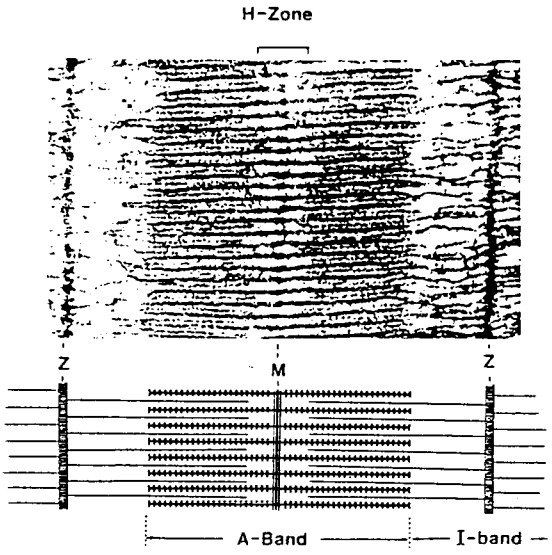

Nematode Obliquelly Striated Muscl

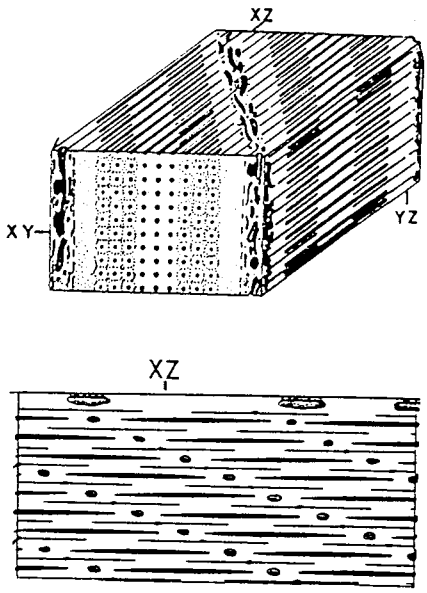

Fig. 5 Obliquily striated muscle

suppressors of unc mutations.

Among these muscle genes, only two have been connected with functional gene products, unc-54 codes for a major heavy chain of myosin (23,24) and unc-15 codes for paramyosin, the core protein of the thick filaments (23,25,26). The unc-54 gene has been cloned and completely sequenced (27,28). Furthermore, over 100 different mutations in the unc-54 gene have been isolated during the course of genetic analysis of muscle-defective strains and 30 of these mutations have been ordered on a genetic fine-structure map (29,30).

In C.elegans, four distinct MHC isoforms, A, B, C, and D, are expressed in a tissue-specific and developmentally regulated manner. MHC A and MHC B are produced in the body wall muscle cells, whereas MHC C and MHC D are synthesized exclusively in the pharyngeal musculature (23,31,32). The C.elegans MHC isoforms can be distinguished by monoclonal antibodies raised against native nematode myosin (33). Immunohistochemical analysis of muscle tissue and isolated thick filaments has shown that MHC A and MHC B are incorporated into different parts of a thick filament. MHC A occupies the central region of each thick filament, while MHC B is present in the lateral portions of the filament. Studies of nummerous mutations that affect the MHC B and produce disorganized body wall muscle have shown that the unc-54 locus encodes MHC B (23,28,33). Three other myosin heavy chain genes, myo-1, myo-2, myo-3, have been isolated from bacteriophage genomic libraries probed with labeled restriction fragments from the unc-54 gene, and it has been demonstrated by monoclonal antibodies that myo-1 encodes MHC D, myo-2 encodes MHC

C, and myo-3 encodes MHC A (34)(Table I).

Structure and Function of Nematode Myosin

In spite of the impressive development of genetic analysis of unc-54 gene, intact myosin had not yet been prepared from C.elegans. We succeeded in purification of intact myosin from C.elegans by our original method (35). In part I, I will show the method of preparation of myosin from C.elegans.

Furthermore, in part III, I will show that myosin preparation obtained by our method contains only one kind of myosin heavy chain isoform which is encoded by unc-54 gene. That is to say, we established the excellent system for investigation of the relationship between structure and function of myosin molecule. Using this system, genetic analysis, mutants and complete amino acid sequence can be utilized in addition to biochemical technique.

Comparison between unc-54 sequence and known sequence of rabbit skeletal myosin shows only 52 % homology between two specimens (36). It is considered that areas of high sequence conservation may correspond to functionally important regions such as those involved in ATP binding and the interaction with actin, while areas of low sequence conservation may reflect the different features of two myosins. Therefore, I have undertaken the following studies using nematode system based on the comparison with rabbit skeletal myosin.

Table. I. Myosin isoform of C.elegans

	Gene	Location	N 2 (wild tipe)	E 675 (mutant)
MHC D	myo-1	Pharynx	206 K	206 K
MHC C	myo-2	Pharynx	210 K	210 K
MHC A	myo-3	Body Wall	210 K	210 K
MHC B	unc-54	Body Wall	210 K	203 K

[1] Submolecular structure of nematode myosin. (part II)

I examined molecular shape and proteolytic digestive pattern of nematode myosin.

[2] Location of flexible hinges in myosin rod. (part II)

A possible flexible hinge, which is supposed elastic element, is predicted from amino acid sequence. I examined the real position of flexible hinges by proteolysis and electron microscopic observations.

[3] Filament formation of nematode myosin. (part III)

The thick filaments in body wall muscle cells are 5 times longer than that of skeletal muscle. I examined whether these different properties are due to myosin alone, or other proteins are necessary.

[4] Heterogeniety of function and structure of two heads of nematode myosin which is composed of unc-54 myosin heavy chain homodimer. (part IV)

[5] Tropomyosin-troponin system in nematode C.elegans. (part V)

The findings from the present study may be helpful in understanding the precise correlation between the primary structure of myosin molecule and its functions. Furthermore, this study may lead to the identification of new genes which encode regulatory proteins involved in muscle contraction.

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Part 1

ATPase Characteristics of Myosin from Nematode *Caenorhabditis elegans* Purified by an Improved Method. Formation of Myosin-Phosphate-ADP Complex and ATP-Induced Fluorescence Enhancement

(SUMMARY)

Myosin was purified rapidly from the nematode Caenorhabditis elegans by an improved method. Crude actomyosin was extracted from the worms at low ionic strength. Paramyosin was removed by repeating the precipitation of myosin filaments in the presence of Mg^{2+} and the dissolutiion of them in 0.6 M NaCl. Actin was removed by ultracentrifugation in the presence of Mg-ATP and finally by column chromatography on DEAE-cellulose. This method gave a good yield of myosin (20-30 mg from 50 g wet weight of worms), and its EDTA(K^+)-ATPase activity was about 3-fold higher than that of myosin prepared by the method of Harris and Epstein (1979) Cell 10, 709-719 . ATP hydrolysis by nematode myosin showed an initial Pi-burst due to formation of the myosin-phosphate-ADP complex. Tryptophan fluorescence of myosin was enhanced about 8% by ATP. The relationship between the structure and function of myosin is discussed based on the above results and the amino acid sequences of myosins from rabbit skeletal muscle and Caenorhabditis elegans.

(INTRODUCTION)

Muscle contraction occurs by a cyclic interaction of myosin with actin coupled with ATP hydrolysis. The structure and function of myosin, the key protein of muscle contraction, have been studied mainly using myosin from rabbit skeletal muscle. However, the amino acid sequence of rabbit skeletal myosin has not yet been completely clarified, and various questions remain as to the structure and function of the myosin molecule (1-5).

The nematode Caenorhabditis elegans has an obliquely striated muscle in its body wall (6). The heavy chain of nematode myosin is mainly encoded by the unc-54 gene (7,8). Recently, the complete amino acid sequence of the unc-54 myosin heavy chain was determined from the DNA sequence (9), and was found to be largely different from that of skeletal myosin (10,11). A large number of mutants in this gene have readily been isolated (7,8,12,13). Therefore, C. elegans is very useful as a model system for studying the structure and function of the myosin molecule.

Myosin has been purified from nematode C. elegans by Epstein et al. (8) and Harris and Epstein (14). However, the myosin prepared by their method did not form the stoichiometric amount of myosin-phosphate-ADP complex, which is the key intermediate of energy transduction in muscle (see 1), and the yield of myosin was not high. In the present study, we found that nematode myosin is not stable in 0.4 M KI which is used to remove actin in the Epstein method (14). Also nematode myosin could easily be dissolved and lost when the nematode homogenate

was washed with a buffer solution at low ionic strength.

The objective of the present study was to develop an efficient method for preparing myosin from nematode C. elegans. Myosin isolated by our new method formed the stoichiometric amount of myosin-phosphate-ADP complex and showed a change in the tryptophan fluorescence upon addition of ATP.

EXPERIMENTAL PROCEDURE

Preparation of Nematode Myosin — The wild-type strain of nematode Caenorhabditis elegans, N2, which was initially isolated by S. Brenner(7), was kindly supplied by Dr. R. Hosono of Kanazawa University. The nematode was synchronously grown on an agar by the procedure of Hosono et al. (personal communication). A Nunc Bioassay dish (22.5 x 22.5 cm) was filled with agar medium (nematode growth medium of (7) except that the concentration of bactopepton was raised to 2.5 %), and a lawn of E. coli bacteria was grown on the agar. Nematodes three days after hatching were applied on the corner of the dish, and they were grown at 20°C for 4 days. Worms were collected by washing the agar surface with 30 ml/dish of buffer containing 0.1 M NaCl and 10 mM K-Pi at pH 6.0. They were starved for 4 h, and then centrifuged at 5,000 x g for 10 min. To remove contaminated bacteria and mold, the pellet was resuspended in 300 ml of 35% (w/v) sucrose and the suspension was immediately centrifuged in a swinging bucket at 3,000 x g for 5 min. The floating nematodes were collected and washed twice with 0.1 M NaCl and 50 mM K-Pi buffer at pH 6.0.

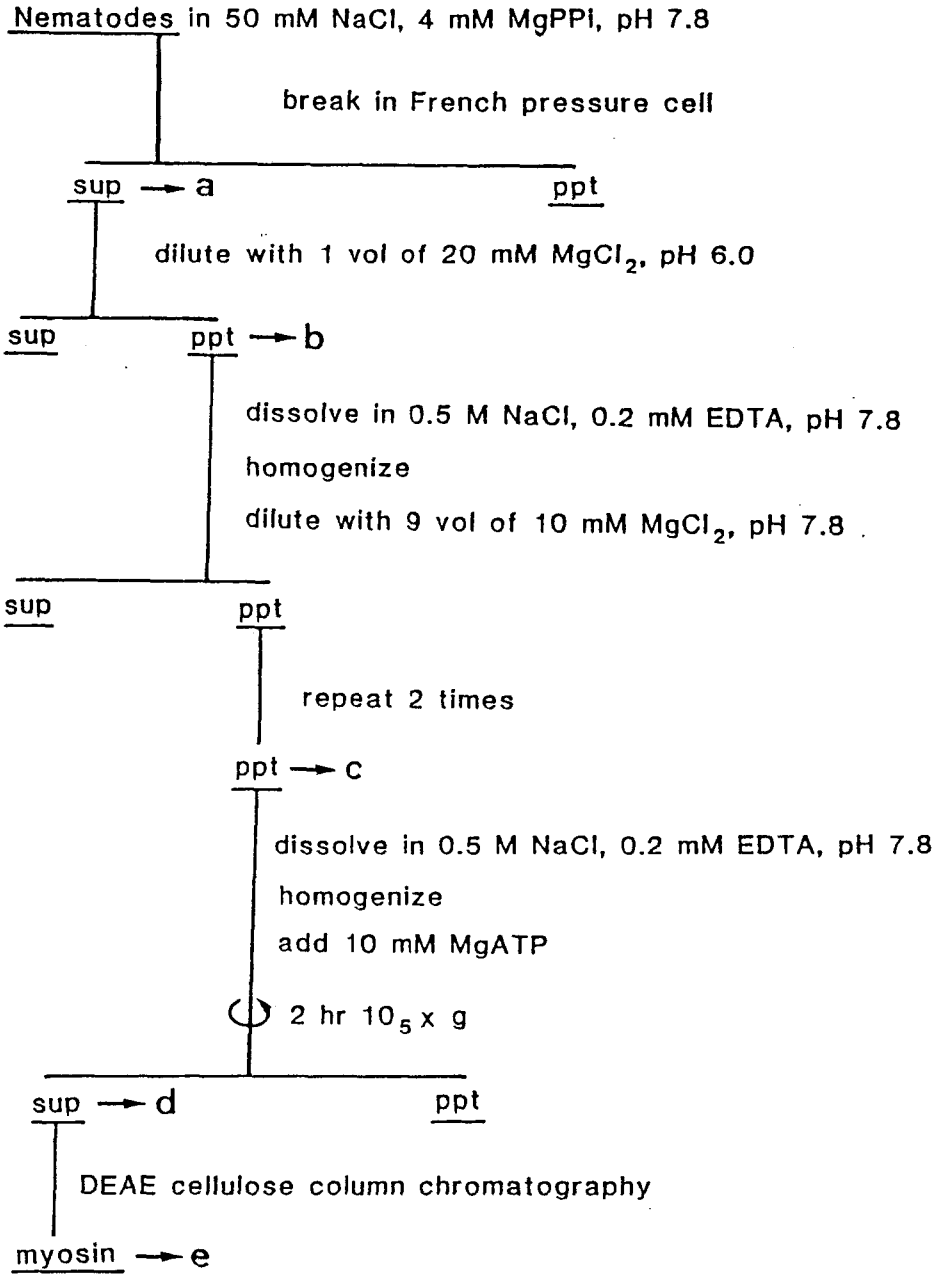

Fig. 1. Scheme for purification of nematode myosin. Fractions a-e were examined by SDS-gel electrophoresis (Fig.2).

About 50 g wet weight of nematodes were obtained from 32 dishes.

Myosin was prepared as diagramed in Fig 1. Allmanipulations were performed on ice or at 4°C and as rapidly as possible to minimize proteolysis. The worms were suspended in 4 vol of extraction solution containing 50 mM NaCl, 4 mM NaPPi, 1 mM EDTA, 3 mM DTT, 1 mM phenylmethylsulfonyl fluoride (PMSF), 50 μM tosyl-L-lysine chloromethyl ketone (TLCK), 20 μg/ml trypsin inhibitor and 20 mM Tris-HCl at pH 7.8, and were centrifuged at 5,000 x g for 15 min. The pellet was resuspended in 8 vol of the same solution. The worms were then homogenized in a precooled French-pressure cell at 8×10^3 lb/in^2. The homogenate was centrifuged at 5,000 x g for 10 min. The supernatant was mixed with an equal volume of buffer containing 20 mM $MgCl_2$, 3 mM DTT, 1 mM PMSF, 50 μM TLCK, 20 μg/ml trypsin inhibitor and 100 mM Tris-maleate at pH 6.0 to form actomyosin filaments. The solution immediately became turbid, and the protein precipitate was collected by centrifugation at 15,000 x g for 20 min. About 20 mg of the protein precipitate (actomyosin fraction) was obtained from each initial gram wet weight of worms. Figure 2A-a and 2A-b show the SDS-polyacrylamide gel electrophoresis (SDS-PAGE) patterns of the initial extract and the actomyosin fraction, respectively, by a Laemmli buffer system (15). The initial extract had about 90% of the myosin heavy chain (210K daltons) contained in a worm. The actomyosin fraction displayed the main bands of the myosin heavy chain (210K daltons), actin (42.5K daltons), and paramyosin (95K daltons).

The actomyosin fraction (precipitate) was dissolved in 0.5 M NaCl, 0.2 mM EDTA, 1 mM DTT, 1 mM PMSF, 50 μM TLCK, 20 μg/ml

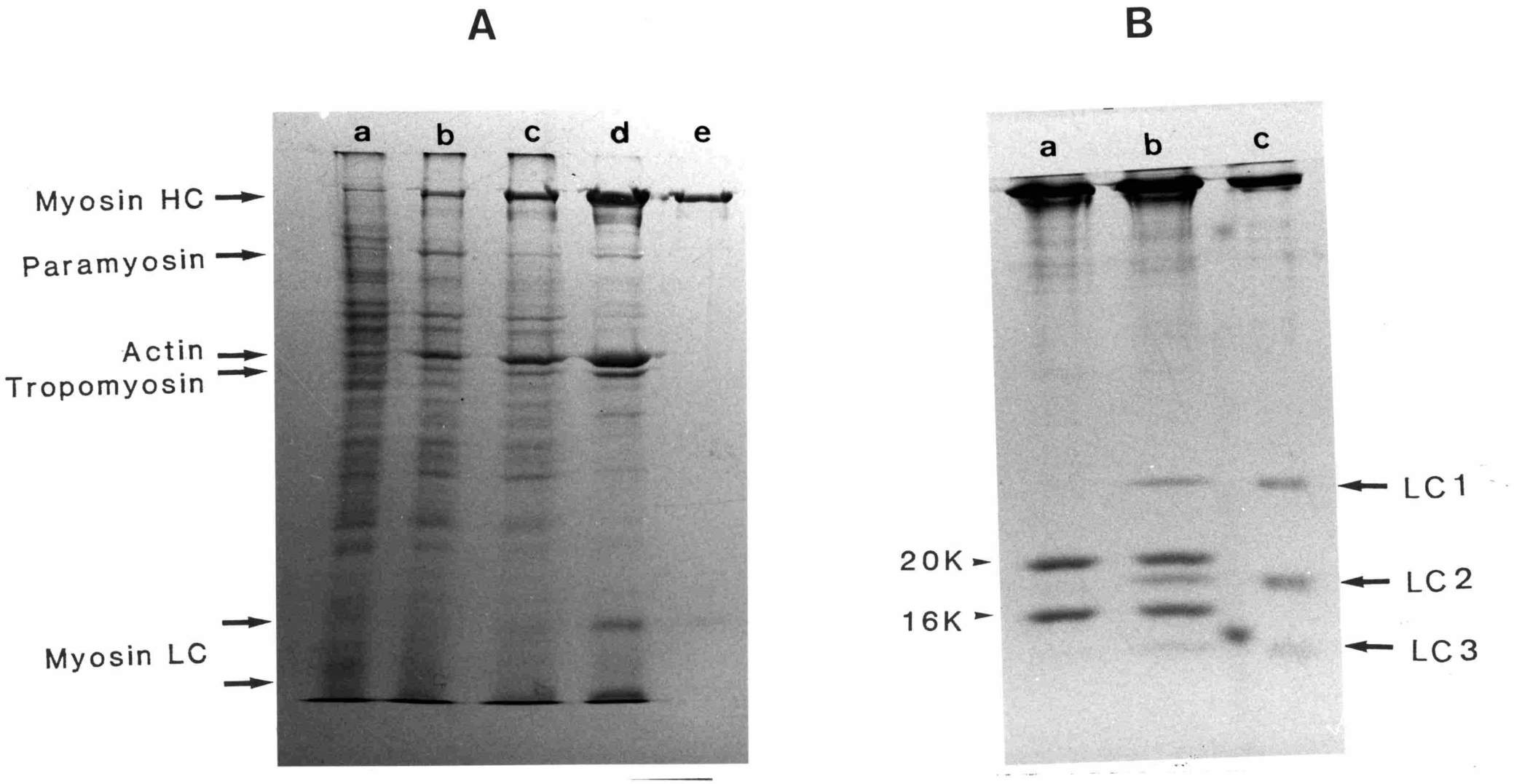

Fig. 2. SDS-gel electrophoretic pattern of nematode myosin at different stages of preparation. A, 10 ug (a-d) or 2 ug (e) total protein of fractions a-e defined in Fig. 1 were run on 12.5 % polyacrylamide gel. B, 10 ug nematode myosin (a), 5 ug rabbit skeletal muscle myosin (c), and mixture of them (b) were run on 15 % polyacrylamide gel.

trypsin inhibitor and 50 mM Tris-HCl at pH 7.8 using a glass homogenizer. The protein concentration of the solution was kept as high as possible, i.e., 3 mg/ml. After diluting with 10 vol of buffer containing 10 mM $MgCl_2$, 1 mM DTT, 1 mM PMSF, 50 μM TLCK, 20 μg/ml trypsin inhibitor and 20 mM Tris-HCl at pH 7.8, the solution was centrifuged at 15,000 x g for 20 min and the precipitate was collected. This step was repeated twice and paramyosin was removed (see Fig 2A-c).

The precipitate was dissolved in 0.6 M NaCl, 10 mM $MgCl_2$, 1 mM DTT, 1 mM PMSF and 10 mM Tris-HCl at pH 7.8 using a glass homogenizer. After adding 10 mM ATP, the solution was centrifuged at 10^5 x g for 2 h and the precipitate was discarded. A large part of F-actin was removed by this procedure. The NaCl concentration of the supernatant was adjusted to 0.18 M by dilution with a buffer containing 0.2 mM ATP, 2 mM $MgCl_2$, 1 mM DTT and 20 mM imidazole-HCl at pH 7.0. Next, the solution, containing 0.8 mg of protein per ml, was applied to a column of DEAE-cellulose (Whatman DE 52) equilibrated with 4 vol of the buffer containing 0.18 M NaCl, 0.1 mM ATP, 2 mM $MgCl_2$ and 20 mM imidazole-HCl at pH 7.0. The volume of the column was more than twice that of the solution applied. The column was eluted with 1 vol of the same solution and then with 2 vol of buffer containing 0.5 M NaCl and 20 mM imidazole-HCl at pH 7.0 at a flow rate of 50 ml/h. The fractions (2 ml) were collected and subjected to determination of protein concentration and SDS-PAGE. As shown in Fig. 3, two protein peaks were obtained, with myosin being eluted in the first peak. Fractions 25-37 were collected and the nucleotide was removed by passing them through a Sephadex G-25

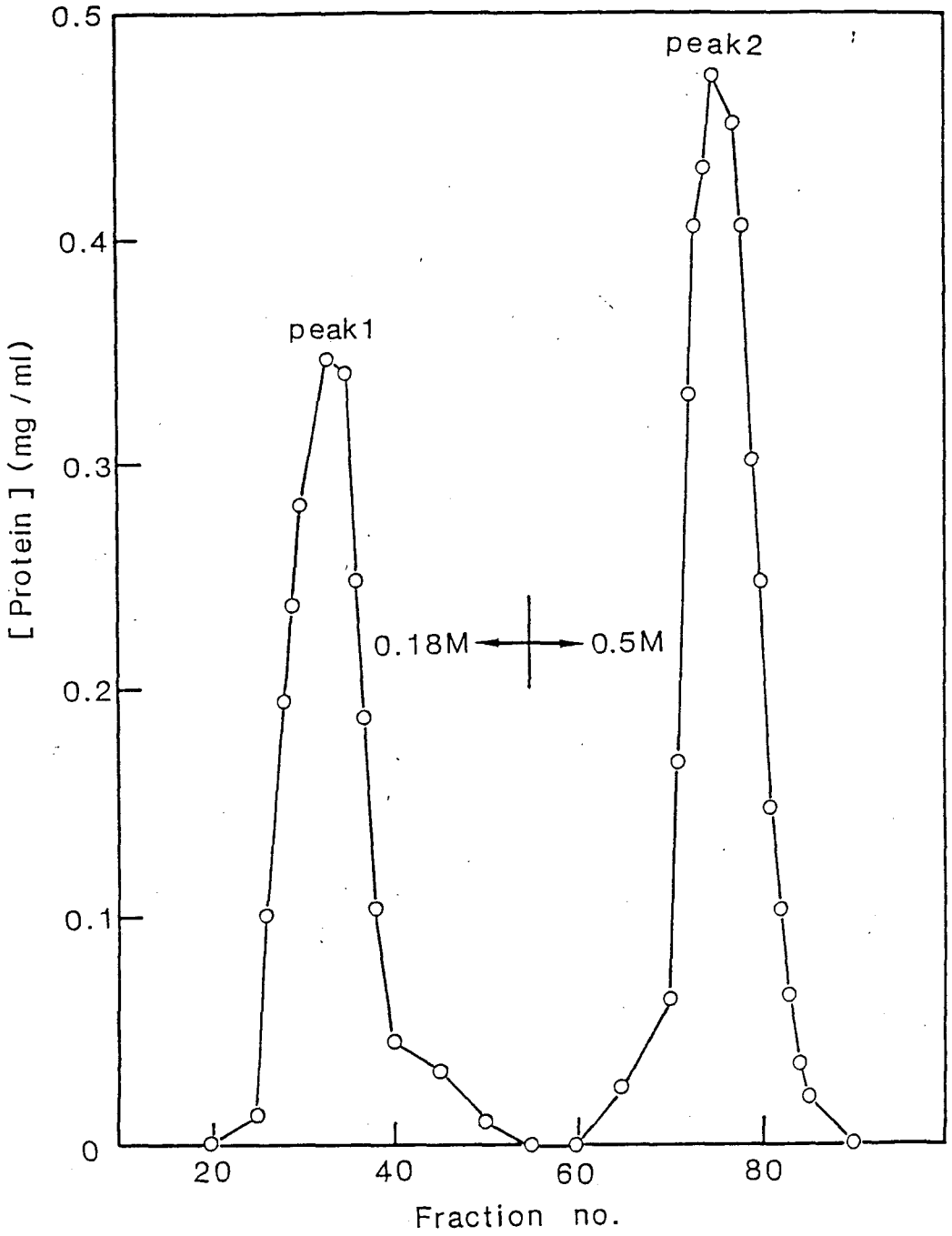

Fig. 3. Elution profile of proteins from DEAE-cellulose column chromatography. Crude myosin (d in Fig.1) was applied to a column (2 x 40 cm) of DEAE-cellulose (Whatman DE-52) equilibrated with 500 ml (4 vol) of buffer containing 0.18 M NaCl, 0.1 mM ATP, 2 mM $MgCl_2$, and 20 mM imidazole-HCl at pH 7.0. The column was eluted with 110 ml of the same buffer and then with 200 ml of the buffer containing 0.5 M NaCl in place of 0.18 M NaCl at a flow rate of 50 ml/h. Fractions of 2 ml each were collected.

column equilibriated with 0.5 M NaCl and 20 mM imidazole-HCl at pH 7.0. As shown in Fig 2A-e, the myosin fraction showed a main protein band with molecular weights of 210K (myosin heavy chain). The area of the myosin heavy chain was more than 80% of the total area. Nematode myosin contained two kinds of light chains (Fig. 2B). Their molecular weights were determined by Weber and Osborn's method (16) to be 20K and 16K, respectively. The yield of myosin was about 0.4-0.6 mg per initial one gram of worms.

Nematode myosin was also obtained by the method of Harris and Epstein (14). Actomyosin was extracted from the precipitate of the homogenate in 0.6 M NaCl. Paramyosin was removed by centrifugation of actomyosin in 0.6 M NaCl at 10^5 x g for 3 h and actin was removed by centrifugation in the presence of ATP at 10^5 x g for 15 min and by KI treatment.

Other Proteins — Skeletal muscle myosin and actin were prepared from rabbit skeletal muscle by the method of Perry (17) and Spudich and Watt (18), respectively. Nematode paramyosin was prepared as described by Harris and Epstein (14). Trypsin inhibitor was purchased from Sigma Chem. Co.

Protein Determination — The concentration of nematode myosin was estimated by the method of Bradford (19), using dried nematode myosin as a standard. The concentrations of skeletal muscle myosin, skeletal muscle actin and nematode paramyosin were estimated by the biuret method, with calibration by nitrogen determination or dry weights.

SDS Polyacrylamide Gel Electrophoresis — SDS polyacrylamide slab gel electrophoresis (SDS-PAGE) (12.5 or 15% acrylamide) was performed using buffer system of Laemmli (15) or Weber and Osborn

(16). Gels were stained with Coomassie brilliant blue.

ATPase Assays — The Ca^{2+}- and EDTA(K^+)-ATPase activities were determined by measuring the rate of Pi liberation according to the method of Youngburg and Youngburg (20). The Ca^{2+}-ATPase activity was measured in 0.02 mg/ml myosin, 1 mM ATP, 0.5 M KCl, 10 mM $CaCl_2$ and 50 mM imidazole-HCl at pH 7.0 and 20°C. EDTA(K^+)-ATPase activity was measured under the same conditions except that 10 mM EDTA was used instead of 10 mM $CaCl_2$. Mg^{2+}-ATPase activity was measured from the time course of ^{32}Pi liberation in 0.2 mg/ml myosin, 0.5 M KCl, 2 mM $MgCl_2$, 20 mM imidazole-HCl at pH 7.0 using 2-8 μM (γ-^{32}P)ATP as substrate. The amount of ^{32}Pi liberated was determined as described previously (21).

Binding of Nematode Myosin to F-Actin — The binding of nematode myosin to F-actin was measured by a centrifugation method. A solution (0.2 ml) of 0.3 mg/ml nematode myosin and 0.6 mg/ml rabbit F-actin in 0.5 M KCl, 2 mM $MgCl_2$ and 50 mM Tris-HCl at pH 7.8 and 0°C was centrifuged at 10^5 x g for 1 h. The supernatant was re-centrifuged after addition of 0.3 mg/ml F-actin, and the resulting supernatant was examined by SDS-PAGE.

Light-Scattering and Fluorescence Measurements — The dissociation and recombination of actomyosin (reconstituted from 0.2 mg/ml nematode myosin and 0.2 mg/ml skeletal actin in 0.5 M KCl, 2 mM $MgCl_2$, 20 mM imidazole-HCl at pH 7.0 and 20°C) after addition of 10 μM ATP were measured from the change in light-scattering intensity at 350 nm using a Hitachi MPF-4 fluorescence spectrophotometer. Myosin fluorescence excited at 295 nm and emitted at 330 nm was measured using the same

apparatus in 0.235 mg/ml myosin, 10 uM ATP, 0.5 M NaCl, 2 mM $MgCl_2$ and 20 mM imidazole-HCl at pH 7.0 and 20°C.

RESULTS

ATPase Activity of Nematode Myosin — Figure 4 shows the time course of ^{32}Pi liberation after 2 μM (γ-^{32}P)ATP was mixed with 0.2 mg/ml (0.4 μM) purified nematode myosin in 2 mM $MgCl_2$, 0.5 M NaCl and 20 mM imidazole-HCl at pH 7.0 and 0°C. About 1 mol of ^{32}Pi per mol of myosin was rapidly liberated in the initial phase of the reaction, and then ^{32}Pi was liberated slowly at a rate of 0.55 min^{-1} (0.0092 s^{-1}). The size of the Pi-burst obtained by extrapolating the time course of ^{32}Pi liberation to time zero was 0.84 mol per mol myosin. The Pi-burst size was not affected when the concentration of (γ-^{32}P)ATP was increased to 8 uM (0.88 mol per mol myosin, data not shown).

To study the possibility of denaturation during preparation we measured the size of the Pi-burst and the myosin content using SDS-PAGE while preparing myosin. The precipitate after dilution (b in Fig. 1) showed a Pi-burst of 0.78-0.85 mol/mol myosin, and its size was not affected even when this fraction had been left for 12 h. We could not accurately measure the myosin content and Pi-burst size for the homogenate of worms or the supernatant after centrifugation of the homogenate (a in Fig. 1). However, the yield and the ATPase activity of purified myosin were not affected by repeating the breakage in the French-pressure cell or leaving the supernatant standing after diluting with 1 vol of

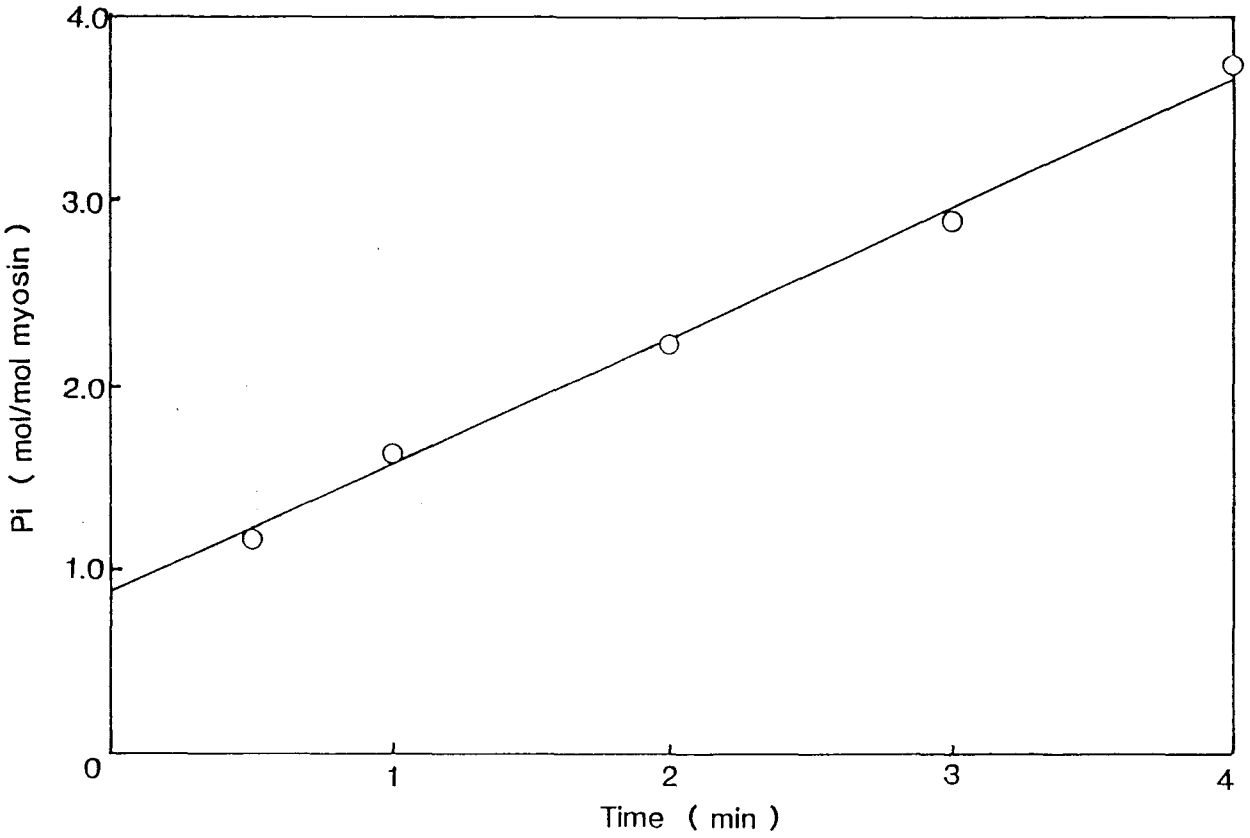

Fig. 4. Time course of Pi liberation in the initial phase of nematode myosin ATPase. The reaction medium contained 0.2 mg/ml nematode myosin, 0.5 M NaCl, 2 mM $MgCl_2$ and 20 mM imidazole-HCl at pH 7.0 and 0°C. The reaction was started by adding 2 μM (γ-^{32}P)ATP and stopped by adding 10% TCA containing 0.1 mM nonradioactive Pi as a carrier.

TABLE I. ATPase activity of nematode myosin. Assay mixture contained 0.5 M KCl, 50 mM imidazole-HCl at pH 7.0. Other conditions are as follows: Ca^{2+}-ATPase, 0.02 mg/ml myosin, 10 mM $CaCl_2$, 1 mM ATP at 20°C; EDTA(K^+)-ATPase, 0.02 mg/ml myosin, 10 mM EDTA, 1 mM ATP at 20°C; Mg^{2+}-ATPase, 0.2 mg/ml myosin, 2 mM $MgCl_2$, 2 μM ATP at 0°C.

	C. elegans myosin (s^{-1})	Rabbit skeletal myosin (s^{-1})
Ca^{2+}-ATPase	0.56 s^{-1}	2.1 s^{-1}
EDTA(K^+)-ATPase	1.02	5.2
Mg^{2+}-ATPase	0.0096	0.0063

20 mM $MgCl_2$ at pH 6.0 for 1 h. The EDTA(K^+)-ATPase activity (ATPase activity in the absence of divalent cations and in the presence of K ions) of purified nematode myosin was of 1.02 s^{-1} (see Table I). The EDTA(K^+)-ATPase activity of purified myosin was not affected by incubation of purified myosin in 10 mM $MgCl_2$ and 20 mM Tris-maleate at pH 6.0 and 0°C for 4 h. When myosin was stored in 0.5 M NaCl and 20 mM imidazole-HCl at pH 7.0 and 0°C for 24 h the Pi-burst size after adding 8 μM ATP decreased from 0.88 to 0.78 mol per mol of myosin.

We studied the ATPase activities of nematode myosin prepared by Epstein's method (Harris and Epstein (14)). This myosin showed a Pi-burst size of 0.1-0.3 mol per mol myosin and EDTA(K^+)-ATPase activity of 0.17 s^{-1}, which was about one-sixth that of myosin prepared by our method (1.02 s^{-1}). As Epstein's method uses 0.6 M KI to remove actin, we studied the effect of KI on the ATPase activity of nematode myosin. The Pi-burst size of purified nematode myosin measured in 0.82 μM nematode myosin, 4 μM (γ-^{32}P)ATP, 0.6 M KCl, 2 mM $MgCl_2$, 1 mM DTT, and 20 mM imidazole-HCl at pH 7.0 and 0°C was 1.1 mol per mol myosin, but it decreased to 0.7 mol per mol myosin when the ATPase reaction was measured immediately after adding 0.6 M KI instead of 0.6 M KCl. When the Mg^{2+}-ATPase reaction was measured in 0.4 M KCl + 0.2 M KI, 0.2 M KCl + 0.4 M KI, or 0.6 M KI at 0°C after incubation for 1 h in each buffer, the Pi-burst size of nematode myosin had decreased to 1.0, 0.24, and 0.1 mol per mol myosin, respectively. The rate of ATPase in the steady state also had decreased from 0.018 s^{-1} (0.6 M KCl) to 0.010, 0.0035, and 0.0015 s^{-1}, respectively (data not shown).

TABLE II. Binding of nematode myosin with F-actin in the presence and absence of ATP. A mixture (0.2 ml) of 0.4 mg/ml nematode myosin and 0.6 mg/ml F-actin in 0.5 M NaCl, 2 mM $MgCl_2$ and 50 mM Tris-HCl at pH 7.8 and 0°C was centrifuged at 10^5 x g for 1 h. The supernatant was re-centrifuged after further adding 0.3 mg/ml of F-actin and the supernatant was examined by SDS-PAGE. The mixture of nematode myosin and F-actin was also centrifuged in the presence of 5 mM ATP, and the supernatant was examined by SDS-PAGE. The amount of myosin and actin were determined from the band intensity after staining of the gel.

	myosin (mg/ml)	actin (mg/ml)
Before centrifugation	0.30	0.60
Supernatant (- ATP)	0.044	0.15
Supernatant (+ 5 mM ATP)	0.26	0.10

The ATPase activity of nematode myosin in the steady state was measured in the presence of Ca^{2+}, EDTA or Mg^{2+} and the data were compared with those of rabbit skeletal myosin (see Table I). Experimental conditions are as described in "MATERIALS AND METHODS". The Ca^{2+}-ATPase activity of nematode myosin was 0.56 s^{-1}, which was about one-fifth that of rabbit skeletal myosin (3.0 s^{-1}). The EDTA(K^+)-ATPase of nematode myosin (1.0 s^{-1}) was also about one-fifth that of rabbit skeletal myosin (4.7 s^{-1}). However, the Mg^{2+}-ATPase activity of nematode myosin (0.0096 s^{-1}) was almost equal to that of skeletal myosin (0.0063 s^{-1}).

Interaction of Nematode Myosin with Skeletal F-actin — The amount of myosin which binds to F-actin was studied by a centrifugal separation method. The mixture of 0.3 mg/ml nematode myosin and 0.6 mg/ml rabbit skeletal F-actin in 0.5 M NaCl, 2 mM $MgCl_2$ and 20 mM imidazole-HCl at pH 7.0 and 0°C was centrifuged at 10^5 x g for 1 h. The supernatant was re-centrifuged after addition of 0.3 mg/ml F-actin, and the supernatant was examined by SDS-PAGE (Table II). The mixture was also centrifuged in the presence of 5 mM ATP. About 80% of actin was precipitated by the centrifugation. Most of myosin (86%) was precipitated in the absence of ATP, and was not precipitated in the presence of ATP.

Figure 5 shows the change in light-scattering intensity of actomyosin after addition of ATP. Actomyosin was reconstituted from 0.2 mg/ml nematode myosin and 0.2 mg/ml skeletal F-actin in 0.5 M NaCl, 2 mM $MgCl_2$ and 20 mM imidazole-HCl at pH 7.0 and 20°C, and 8.5 μM ATP was added as indicated. The light-scattering intensity decreased rapidly after ATP addition, remained constant for about 6 min, and then recovered gradually

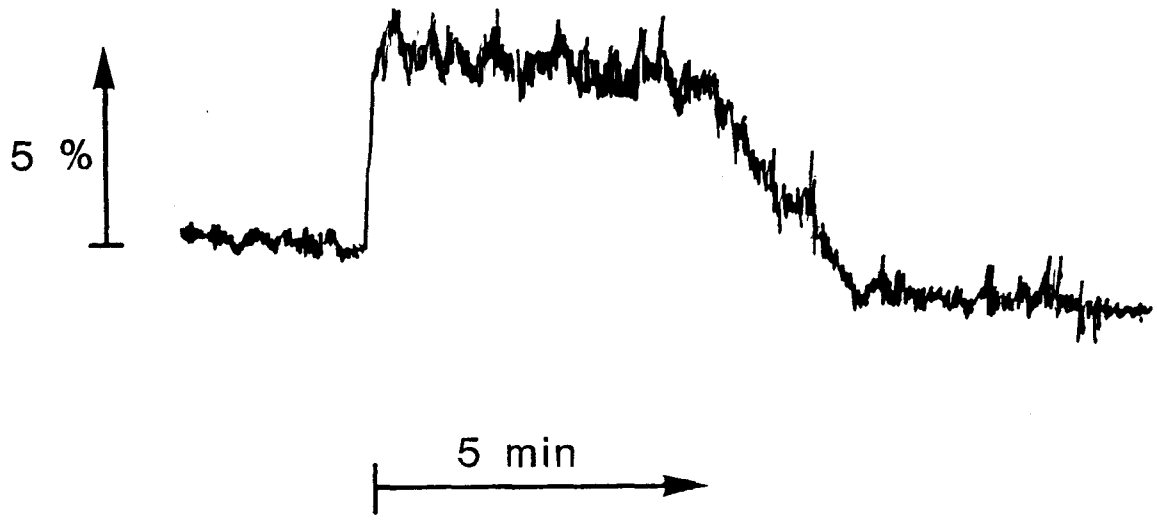

Fig. 5. Time course of change in light-scattering intensity upon addition of ATP to the F-actin-nematode myosin complex. ATP (8.5 μM) was added to a mixture of 0.2 mg/ml nematode myosin and 0.2 mg/ml rabbit skeletal F-actin in 0.5 NaCl, 2 mM $MgCl_2$ and 20 mM imidazole-HCl at pH 7.0 and 20°C.

to the original level. The time required for the recovery was almost equal to that required for complete hydrolysis of ATP, which was estimated from the Mg^{2+}-ATPase activity of myosin (0.55 min^{-1}, Fig. 4).

Change in Fluorescence Intensity of Nematode Myosin Induced by Mg^{2+}-ATP -- The tryptophan fluorescence of nematode myosin was measured in the presence and absence of ATP. Figure 6 shows the fluorescence emission spectrum of nematode myosin excited at 295 nm in the presence (a) or absence (b) of 10 μM ATP. The solutions contained 0.235 mg/ml nematode myosin, 0.5 M NaCl, 2 mM $MgCl_2$ and 20 mM imidazole-HCl at pH 7.0 and 20°C. The emission spectrum of nematode myosin, when exited 295 nm, has its maximum at 334 nm, and the fluorescent intensity exited at 295 nm and emitted at 330 nm was increased by about 8% upon addition of ATP relative to the absolute intensity in the absence of ATP. However, no fluorescence change was detected for myosin prepared by the Epstein's method (14). The inset of Fig. 6 shows the time course of change in fluorescence intensity excited at 295 nm and emitted at 330 nm after 10 μM ATP was added to 0.235 mg/ml nematode myosin under the same conditions. The fluorescence intensity increased rapidly after ATP addition, remained constant level for 5 min, and then decreased gradually to the original level.

DISCUSSION

The nematode Caenorhabditis elegans is very useful for studying muscle contraction because the amino acid sequence of

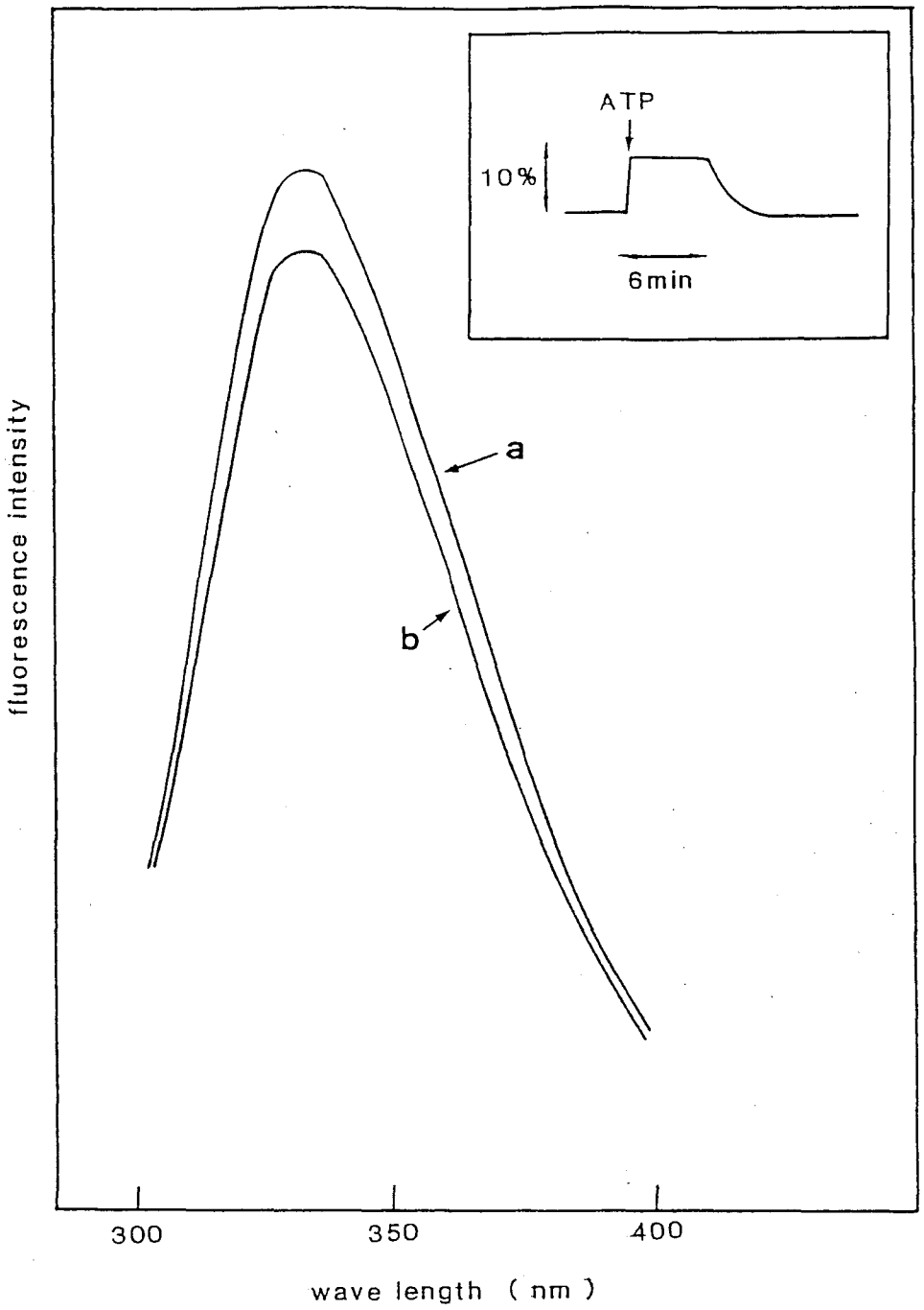

Fig. 6. Enhancement of tryptophan fluorescence of nematode myosin upon addition of ATP. The reaction mixture contained 0.235 mg/ml nematode myosin, 0.5 M NaCl, 2 mM $MgCl_2$ and 20 mM imidazole-HCl at pH 7.0 and 20°C. The excitation wavelength was 295 nm. a, emission spectrum after addition of 10 μM ATP, b, spectrum before addition of ATP. Inset shows the time course of change in fluorescence intensity after addition of 10 μM ATP to nematode myosin under the same conditions. Excitation and emission wavelengthes were 295 nm and 330 nm, respectively.

the myosin heavy chain encoded by unc-54 has been determined from its DNA sequence (9) and various mutants of myosin have been obtained (7,8,12,13). The aim of this study was to examine the ATPase characteristics of nematode myosin especially the Pi-burst due to formation of the myosin-phosphate-ADP complex, M_P^{ADP}, which is the key intermediate in energy transduction in muscle, and also to observe the ATP enhancement of tryptophan fluorescence. Myosin has been obtained from C. elegans by Epstein's group (8,14). But the myosin prepared by their method (14) did not show the stoichiometric amount of Pi-burst or ATP-induced enhancement of tryptophan-fluorescence (see "RESULTS"). Also as they (14) did not study the denaturation of myosin during preparation, some segments of their method adversely affected the myosin sample.

We tried to improve the following three points to increase the yield and quality of myosin. (1) In a Epstein's method (14), the worm homogenate is washed with a medium of low ionic strength, and then myosin is extracted in 0.6 M KCl. However, we found that the filaments of nematode myosin are not stable even at low ionic strength and about 90% of myosin in the homogenate is lost during this washing procedure. Therefore, in our method, the worm homogenate, after French-pressure cell treatment, was centrifuged and myosin was prepared from the supernatant. (2) Epstein's method uses KI to remove F-actin from the actomyosin fraction. However, KI induces denaturation of rabbit skeletal muscle myosin (22,23). We found that ATPase activity and the Pi-burst size of nematode myosin decreased very rapidly in the KI medium (see "RESULTS"). Thus, we remove actin by column chromatography on DEAE-cellulose, since nematode myosin does not

form filament at 0.18 M NaCl in which actin was adsorbed by DEAE-cellulose. (3) The actomyosin fraction contains a large amount of paramyosin. In Epstein's method paramyosin is removed by ultracentrifugation of actomyosin in the absence of ATP at high ionic strength. However, recovering myosin from the actomyosin precipitate is difficult. In the present study, we found that paramyosin could be removed by precipitating actomyosin at low ionic strength in the presence of Mg^{2+} (see Fig. 2).

Before studying the ATPase characteristics of myosin, we checked for possible denaturation of myosin during purification and storage in our method. However, we found that the actomyosin precipitate after dilution with Mg^{2+} solution (b in Fig. 1) showed a Pi burst identical to that of purified myosin (see "RESULTS"). Furthermore, repetitive breakage using a French-pressure cell or leaving homogenate b in Mg^{2+} solution for 1 h did not affect the yield and the ATPase activity of purified myosin. Purified myosin bound strongly to rabbit F-actin but become dissociated from it upon addition of ATP (Figs. 4 and 5). Therefore, we concluded that our method could be useful to obtain intact myosin. The Pi-burst size of purified myosin decreased gradually from 0.88 to 0.78 mol/ mol myosin with storage at 0 C for 24 h. Therefore, we measured the ATPase activity within 10 h after purification.

The nematode Caenorhabditis elegans contains four kinds of myosin heavy chains (two in the body-wall muscle and two in the pharynx) (8). The amino acid sequence has been reported for unc-54 heavy chain in body-wall muscle (9). Since anti-nematode

myosin IgG's is not specific among these four myosins (24,25) the structures of these myosins may be similar. Miller III et al. (25), using monoclonal antibodies against unc-54 and non-unc-54 myosins, showed that non-unc-54 myosin is located in the center of the thick filament and unc-54 myosin on both side of it. Schachat et al. (24) showed that about 60% of myosin in the actomyosin fraction was encoded by the unc-54 gene by using the specific antibody. However, they (24) extracted actomyosin from the homogenate after washing it with a buffer of low ionic strength which dissociates most of the myosin in the homogenate ("EXPERIMENTAL PROCEDURES"). Because we purified myosin from the supernatant of the homogenate, the unc-54 myosin content in our myosin preparation should be higher that (60%) reported by Schachat et al. (24).

Since the amino acid sequence of nematode myosin (9) is quite different from that of rabbit skeletal muscle myosin (10,11), we tried to find, whether the two myosins have the same properties. We found that nematode myosin forms the myosin-phosphate-ADP complex (M_P^{ADP}) by reaction with Mg-ATP. In the case of skeletal muscle myosin, the two heads of myosin have different structure and functions and M_P^{ADP} is formed only in one of them, with the myosin-ATP complex (M-ATP) being formed in the other (1). The amount of M_P^{ADP} can be determined from the size of the Pi-burst since M_P^{ADP} decomposes when the reaction is stopped by TCA. The size of the Pi-burst for nematode myosin was also 1 mol per mol of myosin (see Fig. 6). Therefore, nematode myosin also seems to have nonidentical heads.

The EDTA(K^+)-ATPase activities of bovine arterial (26),

chicken gizzard (27), and cardiac muscle myosin (28) have been reported to be similar to that of skeletal muscle myosin. However, the EDTA(K^+)-ATPase and Ca^{2+}-ATPase activities of nematode myosin were about one-third those of skeletal muscle myosin (TABLE I). This is not due to denaturation of myosin, since the Pi-burst size of nematode myosin was the same as that of skeletal muscle myosin. We assumed that this may reflect a difference in the amino-acid sequence of ATP-binding peptide. The complex of nematode myosin and F-actin showed typical superprecipitation when ATP was added at low ionic strength (data not shown). The actin activated ATPase activity will be described in a following paper (Tanii, Osafune, Arata & Inoue, in preparation).

Skeletal muscle myosin shows the tryptophan-fluorescence enhancement induced by ATP (31). We found that the fluorescence intensity of nematode myosin was also enhanced by the addition of ATP (Fig. 7). The extent of fluorescence enhancement of nematode myosin (8%) was lower than that (11%) of skeletal muscle myosin. Werber et al. (29) suggested that about 2 tryptophan residues in the two heads of myosin are buried when myosin reacts with ATP. The heavy chain of myosin head with molecular weight of 100k is digested by trypsin into 3 segments with molecular weight of 25K, 50K, and 21K (30). ATP photoaffinity labels bound covalently to the 25K (31) or 50 K segment (32), while actin was cross-linked with 50k and/or 21K segments (33). The tryptophan residue which induces fluorescence change was considered to be located in the N-terminal 25K segment since an ATP photoaffinity label binds covalently to the specific tryptophan residue on the 25K peptide

(34). However, since there is no common tryptophan residue in the N-terminal 25K-dalton peptide between unc-54 myosin (9) and rabbit skeletal muscle myosin (11), the fluorescence change of myosin may not be due to the specific residues on the 25K-dalton peptide.

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Part 2

Submolecular Structure of Myosin Molecule from the Nematode *Caenorhabditis elegans*

SUMMARY

The submolecular structure of myosin prepared from the nematode Caenorhabditid elegans has been examined by electron microscopy using a rotary shadow-casting technique and formation of subfragments by proteolytic digestion. The electron micrograph of nematode myosin which have been rotary-shadowed with platinum has similar appearance to rabbit skeletal muscle myosin, i.e., two globular heads attached to a long tail. The diameter of head was 18 nm and the length of tail was 152 nm. The tail was bent sharply at a position of 84 nm from the head-tail junction. Heavy meromyosin was obtained by chymotryptic digestion in the presence of Mg^{2+}. The molecular weight of HMM heavy chain was estimated from SDS-polyacrylamide gel electrophoresis to be 140 K. The electron microscopy of HMM resembled a two-headed structure with tail length of 55 nm. Subfragment-1 with molecular weight of 100 K was obtained by papain digestion of myosin in the presence of EDTA. These results suggested that head-tail junction and S-2-LMM junction are flexible and suspectible against protease even in nematode myosin. However, the position of S-2-LMM hinge determined by the proteolysis (55 nm from head-tail junction) and the bending position (82 nm) were different from that predicted from the amino acid sequence (49 nm).

(Introduction)

Muscle contraction occurs by a cyclic interaction between myosin head which composes projections from the thick filament and actin which is the main component of thin filament. The myosin molecule has special structure based on its function. When myosin molecule is obserbed by electron microscopy after shadow-casting using the mica-replication or by negative staining, myosin has two globular heads connected to a long fibrous tail (1-3). Proteolytic digestions of myosin results the separation of the molecule into functionally distinct parts (4,5). There are two susceptible regions in the molecule, the junction between head and tail, and center of the tail. These regions are flexible when the molecule was observed by electron microscopy (2,3). When myosin was digested by trypsin, HMM and LMM are formed (4). When HMM was digested further by trypsin, S-1 and S-2 are formed (5). Wereas when myosin was digested by chymotrypsin or papain, S-1 and rod (tail) are formed (6,7). S-1 and HMM are water-soluble even at low ionic strength and retain ATPase and actin binding activity (4,5). Rod and LMM have a two-stranded coiled-coil α-helical structure and aggregate at low ionic strength (4). Therefore, backbone of thick filament is formed by LMM part. The head part (S-1) is connected with LMM through S-2, and the junctions between S-1-S-2 and S-2-LMM are flexible. Therefore, S-2 part is considered to play the role of a crunk shaft. Furthermore, S-2 region near the junction between HMM and LMM is considered to be an elastic component of crossbridge (8).

The amino acid sequence of whole myosin molecule has been reported on a nematode caenorhabditis elegans from the DNA-sequence (9,10). However, the sequence of nematode myosin are greatory different from known region of skeletal muscle (11,12). Recently, we have obtained unc-54 rich myosin from C.elegans (3). Then, in this paper we studied the submolecular structure of the myosin molecule both by electron microscopy and by proteolytic digestion. The results obtained were compared eith the amino acid sequence of nematode myosin.

EXPERIMENTAL PROCEDURE

Proteins-Nematode C.elegans myosin was prepared as described in the previous paper (13). Skeletal muscle actin was prepared from an aceton powder of rabbit skeletal muscle by method of Spudich and Watt (14). Papain and chymotrypsin were purchased from Sigma Chemical Co. LTD.

Preparation of Myosin Subfragment-HMM was obtained by the chymotryptic digestion of nematode myosin essentially the same method as described by Weeds and Taylor (15). Myosin (0.5 mg/ml) was digested with 1/20 W/W (0.025 mg/ml) of chymotrypsin in 0.5 M NaCl, 10 mM $MgCl_2$ and 20 mM imidazole-HCl at pH 7.0 and 15°C for 10 min. The digestion was terminated by adding of 0.1 mM phenylmethylsulphonyl fluoride (PMSF). The solution was dialyzed against the buffer containig 50 mM NaCl, 10 mM $MgCl_2$, 1 mM DTT, 20 mM imidazole-HCl at pH 7.0 and 4 C for 12 h. The digest was

centrifuged at 10^5 x g for 1 h, and the supernatant was used as HMM fraction.

S-1 was obtained by papain digestion of myosin (6). Myosin (0.5 mg/ml) was digested with 1/20 W/W (0.025 mg/ml) of papain in 0.5 M NaCl, 10 mM EDTA, 20 mM imidazole-HCl at pH 7.0 and 20°C for 10 min. The digestion was terminated by adding of 0.5 mM N-p-tosyl-lysine chloromethylketon (TLCK) and 0.25 mM iodoacetic acid. the solution was dialyzed against the buffer containig 50 mM NaCl, 10 mM $MgCl_2$, 1 mM DTT, and 20 mM imidazole-HCl at pH 7.0 and 0 C, and then the solution was centrifuged at 10^5 x g for 1 h, and the supernatant was used as S-1 fraction. S-1 fraction (0.4 mg in 1 ml) was applied to a column (1.0 x 13 cm) of Sephacryl S-300, equilibrated with a column buffer containing 0.5 M NaCl, 0.5 mM EDTA, 1 mM DTT and 20 mM imidazole-HCl at pH 7.0. The column was eluted with the column buffer at a flow rate of 10 ml/h, and the fractions were collected.

Protein Determination-The concentration of nematode myosin was estimated by the method of Bradford (16), using dried myosin as a stsndard. The concentrations of myosin fraction were also measured by the same method. The concentration of skeletal muscle actin was estimated by the biuret reaction calibrated by nitrogen determination or dry weight.

SDS-Polyacrylamide Gel Electrophoresis-SDS-polyacrylamide slab gel electrophoresis (SDS-PAGE) (10 % acrylamide) was performed using the buffer system of Laemmli (17). Gels were stained with Coomassie Brilliant Blue.

Mg^{2+}-ATPase Assay-Mg^{2+}-ATPase activity was measured from the time course of ^{32}Pi liberation using (γ-^{32}P) ATP as substrate.

Myosin (0.06 mg/ml) or its proteolytic digest was mixed with 5 μM (γ-^{32}P) ATP in 0.5 M NaCl, 5 mM $MgCl_2$, and 20 mM imidazole-HCl at pH 7.0 and 0°C. The reaction was terminated by adding 5 % TCA and the amount of ^{32}Pi liberated was determined as described previously (18).

Electron Microscopy-Rotaly-shadowed specimens for an electron microscopy were prepared according to the method of Eliott and Offer (2). Proteins were dialyzed at a concentration of 0.1 to 0.2 mg/ml against 0.6 M ammonium acetate at pH 7.2. Before spraying, the proteins were diluted with 0.6 M ammonium acetate solution containing 30 % (v/v) glycerol to give a protein concentration of 0.01 to 0.03 mg/ml. Imidiately droplet were sprayed into freshly cleaned mica and dried in vacuo at room temparature for 3 h by which time the vacuum achieved was about 1.5×10^{-6} torr. The dried surface was rotary-shadowed with platinum at an angle of 1 in 10. The replica was examined using a JEOL 100S electron microscope at 80 kv.

RESULTS

The Shape of C.elegans Myosin-Figure 1 shows a field of myosin molecules obtained from the nematode Caenorhabditis elegans. The sample was rotary-shadowed with platinum after drying from a droplet containing glycerol and platinum replica was examined by electron micropy. The molecules have two heads attached to a long tail. The general appearance of molecule is quite similar to that of skeletal muscle myosin. The head of

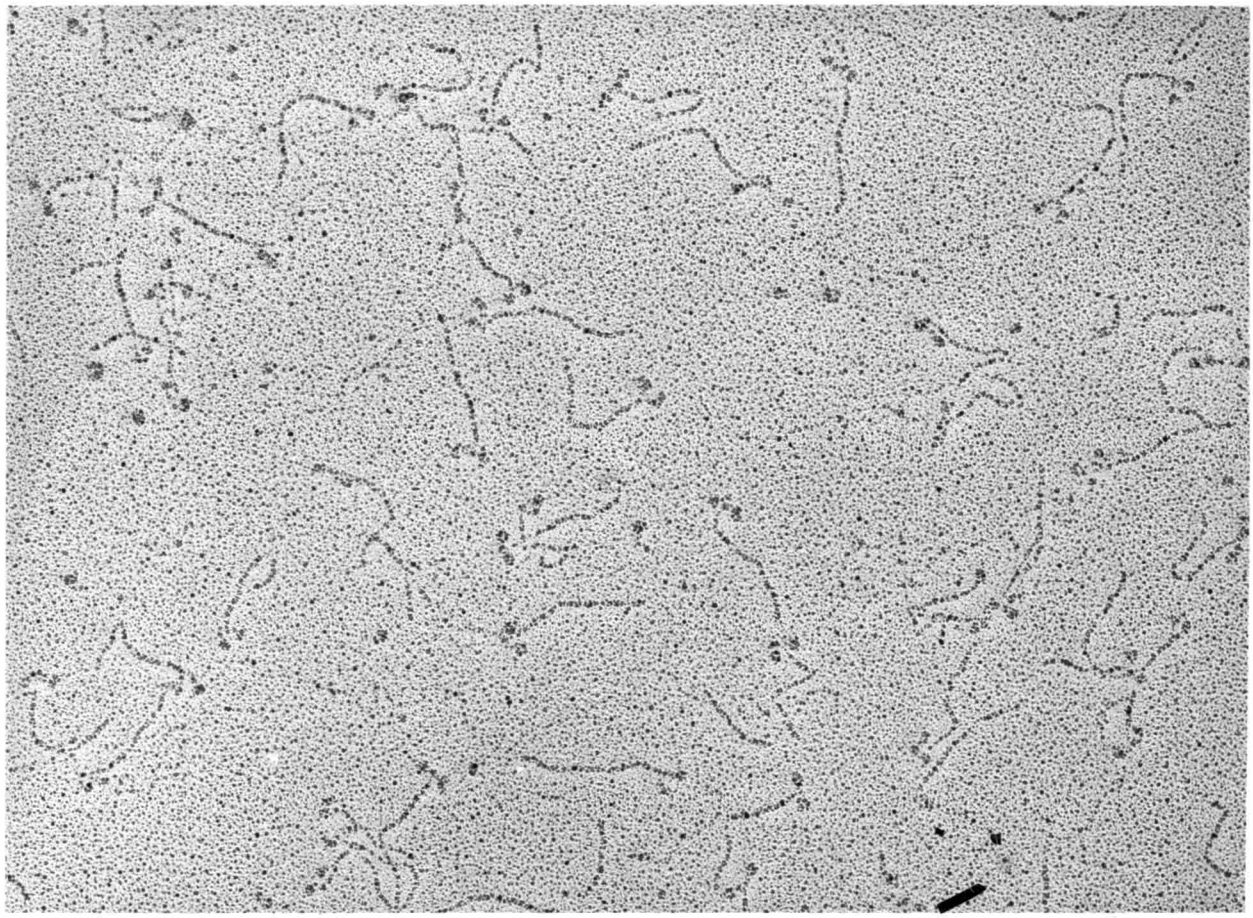

Fig. 1. Nematode myosin molecules rotaly-shadowed with platinum. The myosin (0.05 mg/ml) was dried from 0.6 M ammonium acetate and 30 % (v/v) glycerol at pH 7.2. Magnification: 115,000 x. Scale bar is 100 nm.

nematode myosin was about 18 nm in diameter and they were spherical or a little elongated. The distribution of measurments of tail lengths had a peak between 150 and 155.6 nm.

Chymotryptic Digestion of Myosin-Nematode myosin (0.5 mg/ml) was digested with 1/20 w/w (0.025 mg/ml) of chymotrypsin in 0.5 M NaCl, 10 mM $MgCl_2$ and 20 mM imidazole-HCl at pH 7.0 and 20°C for 10 min. As shown in Fig. 2, a and b, about 70 % of myosin heavy chain was digested. The chymotryptic digest was dialyzed against 50 mM NaCl and then centrifuged at 10^5 x g for 1 h. The superntant mainly contained 350 K molecular weight protein when analyzed by Sephacryl S-300 column chromatography. The SDS-PAGE revealed that this fraction contained 150 K peptide and small amount of 75 and 60 K peptide (Fig. 2 c). These proteins precipitated with F-actin (Fig. 2, d and e). Therefore, the supernatant is mainly composed of HMM. The precipitate (LMM fraction) contained 49 and 46 K peptidealong with 210 K peptide. Figure 3 shows the shape of HMM molecules which have been rotary-shadowed with platinum. The shape of HMM resenbled that of myosin (Fig. 1), but the length of tail of HMM molecule was 55 nm which is about one third of that of myosin.

When HMM was centrifuged in the presence of F-actin in 0.5 M NaCl, 10 mM $MgCl_2$ and 20 mM imidazole-HCl at pH 7.0 and 20°C at 10^5 x g for 1 h, HMM was cotained in the precipitate (Fig. 2 d), but ATP induced the dissociation of HMM from F-actin (Fig. 2 e). Figure 4 shows the time course of ^{32}Pi liberation in the Mg^{2+}-ATPase reaction of myosin and its chymotryptic digest. The size of Pi burst of digested myosin was 0.78 mol per mol myosin, which was same as that of undigested myosin. Mg^{2+}-ATPase

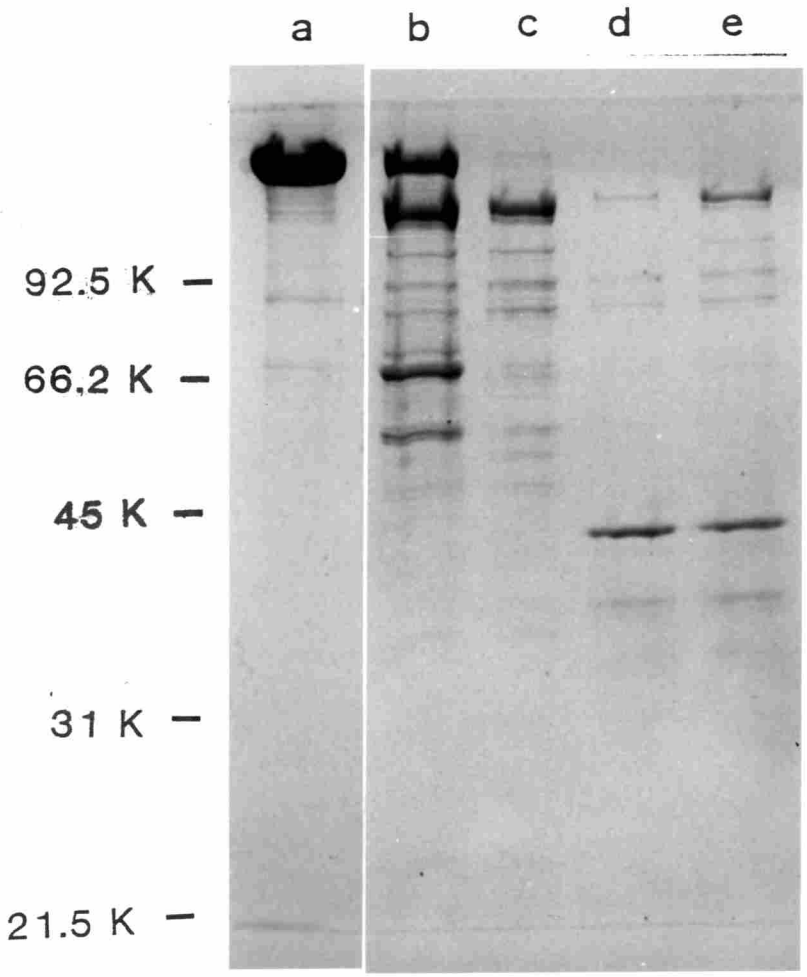

Fig. 2. SDS-gel electrophoretic analysis of the chymotryptic digestion of nematode myosin. 10 % polyacrylamide gel. (a) myosin, (b) chymotrypyic digest of myosin, (c) HMM, (d) supernatant after centrifugation of acto-HMM, (e) supernatant after centrifugation of acto-HMM in the presence of ATP.

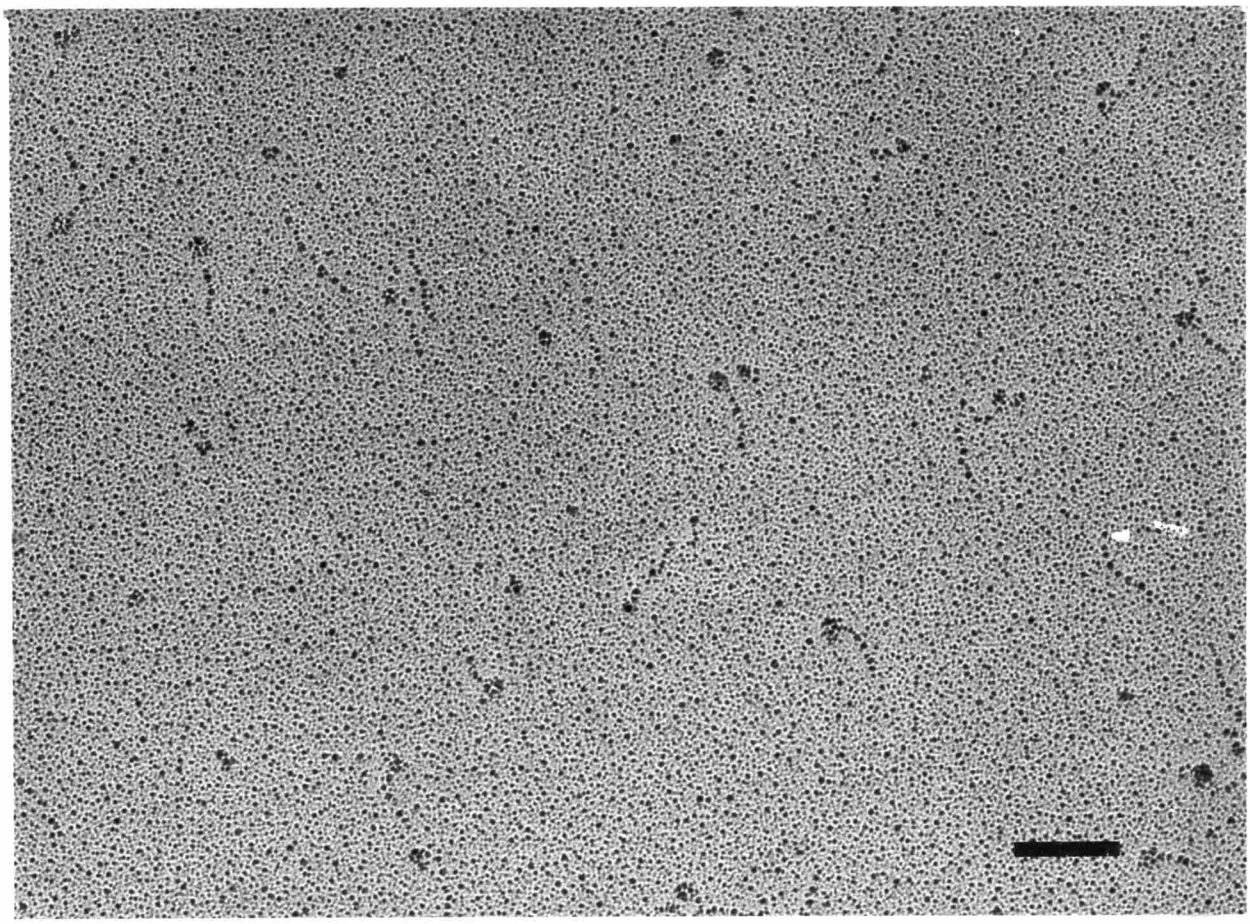

Fig. 3. HMM obtained from nematode myosin rotary-shadowed with platinum. HMM (0.02 mg/ml) sample was dried from 0.6 M ammonium acetate and 30 % (v/v) glycerol at pH 7.2. Magnification: 153,000 x. Scale bar is 100 nm.

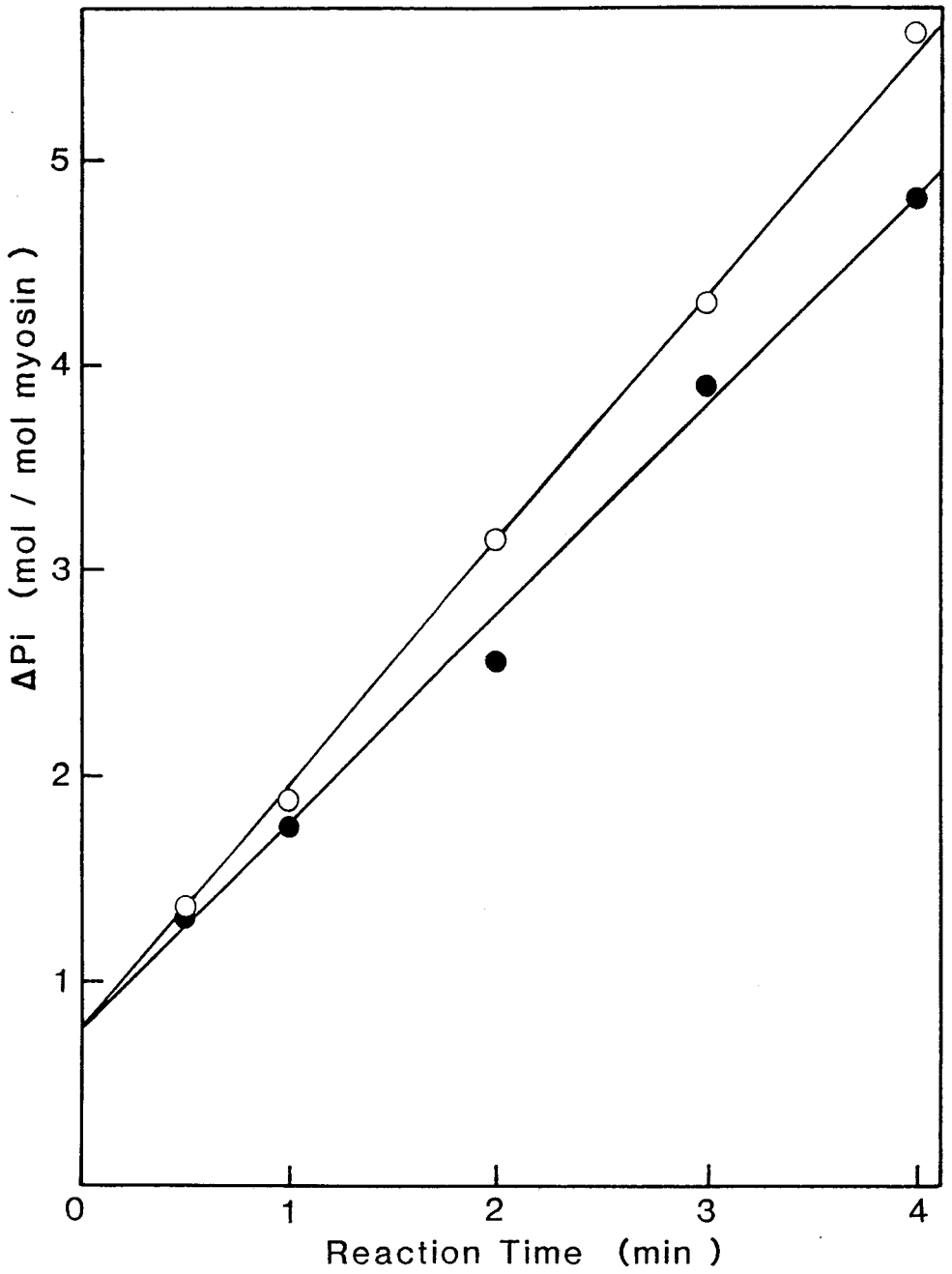

Fig. 4. Effect of chymotryptic digestion of myosin on the Mg^{2+}-ATPase reaction. The reaction medium contained 0.12 mg/ml myosin (○) or its chymotryptic digest (●) in 0.5 M NaCl, 2 mM $MgCl_2$ and 20 mM imidazole-HCl (pH 7.0) and 0°C. The reaction was started by adding 5 μM (γ-^{32}P)ATP and stopped by adding 10% TCA containing 0.1 mM non-radioactive Pi as a carrier.

activity of digested myosin in the steady state was 0.020 s^{-1}, which was slightly lower than that of undigested myosin (0.023 s^{-1}).

When myosin (0.5 mg/ml) was digested with 1/20 w/w (0.025 mg/ml) of papain in 0.5 M NaCl, 10 mM EDTA and 20 mM imidazole-HCl at pH 7.0 and 20°C for 10 min. About 95 % of myosin heavy chain was digested by this treatment (Fig. 5, a and b). After dialyzing against the buffer containing 50 mM NaCl, 10 mM $MgCl_2$ and 20 mM imidazole-HCl at pH 7.0 and 0°C, the digest was centrifuged at 10^5 xg for 1 h. The SDS-PAGE pattern of digested myosin showed that the papain digest cotained 95 K peptide mainly and small amount of 130, 80, 75, 60 and 55 K peptide (Fig. 5 b). Only 90 K peptide was found in the supernatant after centrifugation (Fig. 5 c). Figure 6 shows the elution profiles of myosin and S-1 fraction from Sephacryl S-300 column. Myosin was eluted at the position 500 K. The column chromatography of S-1 on Sephacryl S-300 revealed that S-1 fraction is mainly composed protein with molecular weight of 100 K. Figure 7 shows the molecular shape of S-1 examined by electron microscopy after rotary-shadowing with platinum. S-1 has a ball like shape with diameter of about 18 nm, which was almost the same as that of heads part of myosin.

When acto-S-1 was centrifuged at 10^5 x g for 1 h, S-1 was precipitated with F-actin (Fig. 5 d), while in the presence of 10 mM ATP, the acto-S1 complex dissociated and S-1 was recovered in the supernatant (Fig. 5 e). Figure 8 shows the time course of ^{32}Pi liberation in the Mg^{2+}-ATPase reaction of myosin and the papain digest. The time course of ^{32}Pi liberation by papain

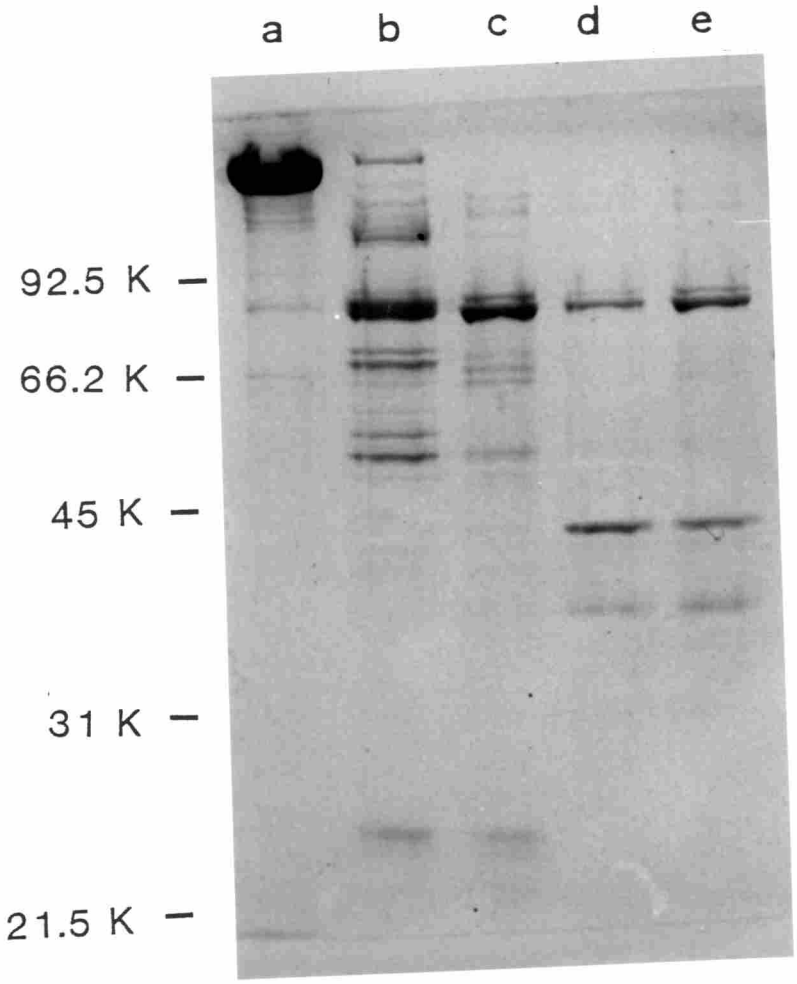

Fig. 5. SDS-gel electrophoretic analysis of papain digestion of nematode myosin. 10 % polyacrylamide gels. (a) myosin, (b) papain digest of myosin, (c) S-1, (d) supernatant after centrifugation of acto-S-1, (e) supernatant after centrifugation of acto-S-1 in the presence of ATP.

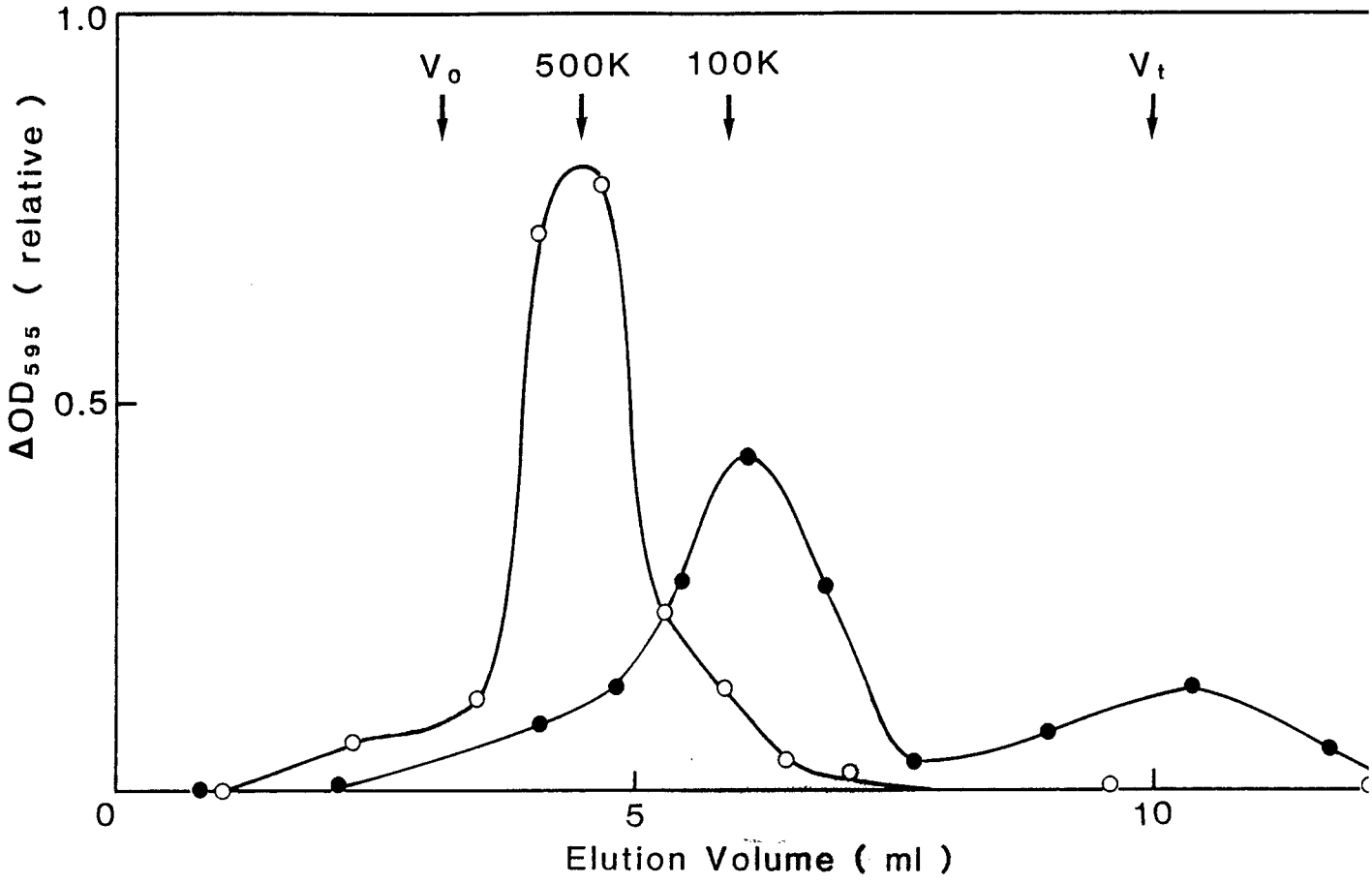

Fig. 6. Elution profiles of myosin and S-1 from Sephacryl S-300 column chromatography. Myosin (0.59 mg) (○) and S-1 (0.39 mg) (●) were applied to the column (1.2 x 13 cm) of Sephacryl S-300 equilibrated with the buffer containing 0.5 M NaCl, 0.5 mM EDTA, 1 mM DTT and 20 mM imidazole-HCl (pH 7.0), and eluted by the same buffer. The void volume of the column (V_0 = 3 ml) was determined with Blue Dextran 2000. V_t shows the column volume.

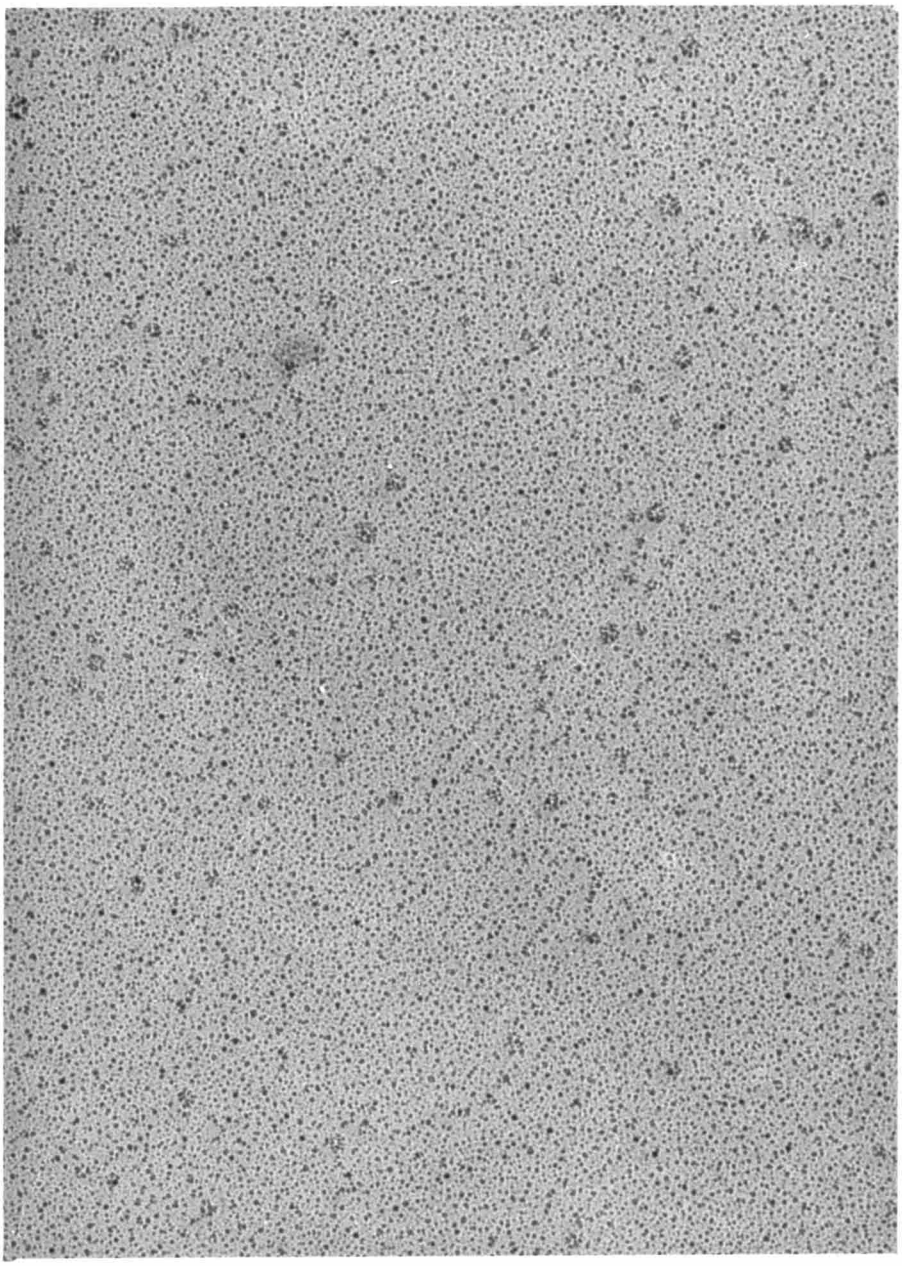

Fig. 7. S-1 obtained from nematode myosin, rotary-shadowed with platinum. S-1 (0.02 mg/ml) was dried from 0.6 M ammonium acetate and 30 % (v/v) glycerol at pH 7.2. Magnification: 135,000 x. Scale bar is 100 nm.

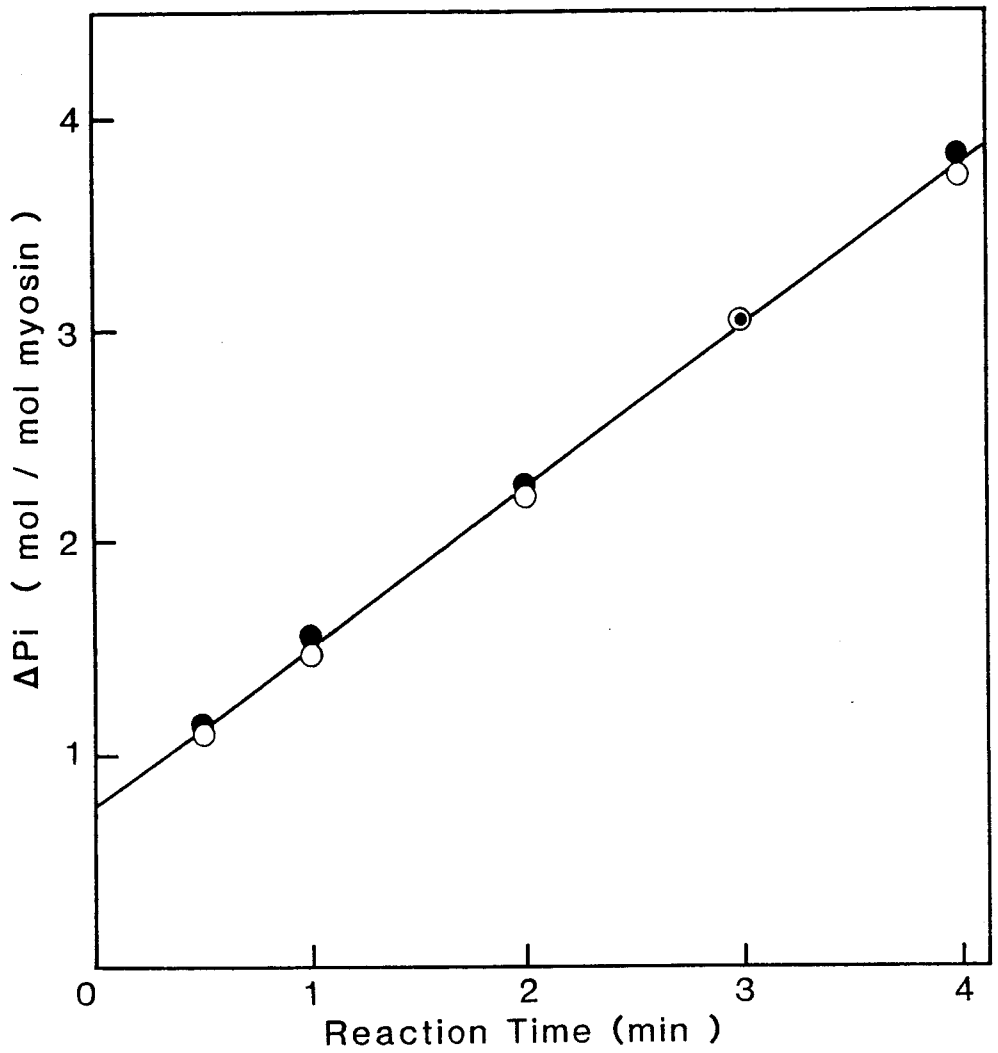

Fig. 8. Effect of papain digestion of myosin on the Mg^{2+}-ATPase reaction. The reaction medium contained 0.11 mg/ml myosin (○) or its papain digest (●). Other conditions are as described in Fig. 4.

digest was the same as that of undigested myosin. The sizes of Pi burst were 0.7 mol per mol myosin and the Mg^{2+}-ATPase activities in the steady state were 0.016 s^{-1}.

DISCUSSION

The nematode Caenorhabditis elegans has four myosin heavy chain genes (19). The body wall muscle myosin is mainly encoded by unc-54 gene which DNA sequence has already known (10). In the previous paper (13) we prepared myosin from the nematode C. elegans by an improved method. This myosin is mainly composed of unc-54 myosin. Therefore, in the present paper we studied the molecular shape and flexibility of myosin by electron microscopy after rotary-shadowing and by proteolytic digestion of myosin into subfragment, and the results were compared to the amino acid sequence of myosin.

The shape of nematode myosin molecule is almost the same as rabbit skeletal myosin described by Elliot & Offer (2). It has two separated heads with a long tail, but there is a little difference between the two specimen. The head of nematode myosin is spherical, whereas rabbit skeletal myosin has rather elongated pear-shaped heads (2). It was shown by Flicker et al. (20) that the two heads of scallop myosin was pear-shaped but the head appear to be round and shorter by removal of regulatory light chain. However, the nematode myosin has two kinds of light chains (13). Therefore, the structure of head tail junction is not stable and shrinked in the nematode myosin.

The tail of myosin is 152 nm in length which is 10 nm shorter than the length estimated from amino acid sequence (162 nm), and also slightly shorter than that of skeletal muscle myosin (156 nm). Therefore, the tail of nematode myosin is not completely an -helical dimer.

In skeletal muscle myosin head-tail junction is flexible and susceptible against protease. This flexibility is considered to be important for generation of tension and constraction. In the nematode myosin head-tail junction was also flexible (Fig. 1) and S-1 (head) was produced by papain digestion of myosin in the presence of EDTA (Fig. 5). S-1 retained the ATPase activity and actin-binding ability (Fig. 5-8). This head-tail junction of nematode myosin was similar to those of skeletal myosin though the amino acid sequence of nematode myosin are different from that of skeletal myosin (10). Papain digestion of myosin produced 95,000 dalton peptide corresponding to S-1 heavy chain and 130,000 dalton peptide corresponding to rod. The molecular weights of S-1 heavy chain and rod were slightly higher than that of skeletal muscle (90,000 and 120,000, respectively). Molecular weight of S-1 heavy chain deduced from amino acid sequence is 95,392 daltons (10), which is consistent with the present result (Fig. 5).

The chymotryptic digestion of soluble myosin in the presence of Mg^{2+} produced 150,000 dalton peptide corresponding to HMM heavy chain, and 75,000 and 60,000 dalton peptides corresponding to LMM. HMM is soluble even at low ionic strength and retains the ATPase activity and actin-binding activity while LMM assembled into filament at low ionic strength.

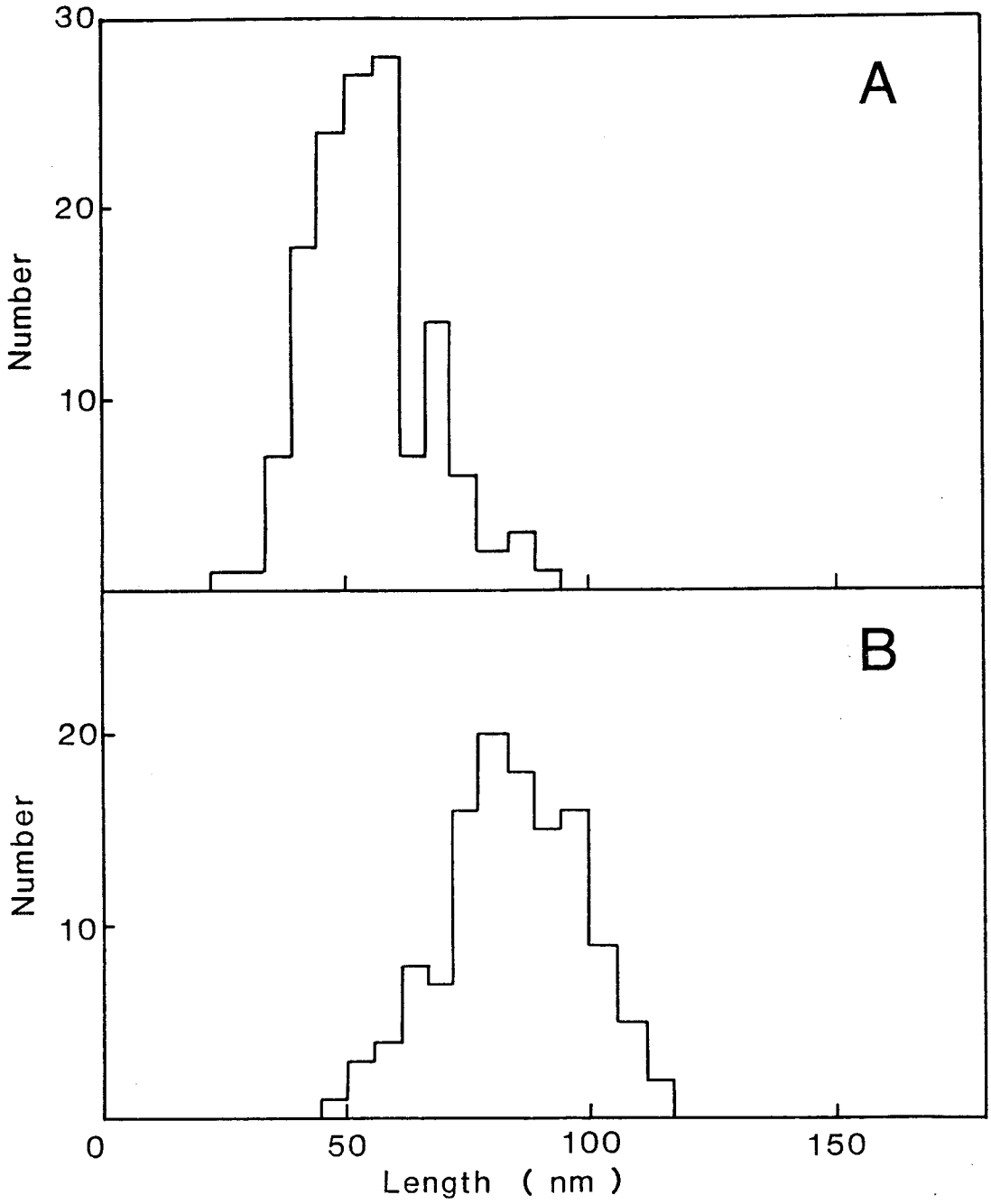

Fig. 9. Histograms defining the length of the tail of HMM and the position of the hinge in myosin tail. A. total length of the tail of HMM measured by electron microscopy (Fig. 3). The length from head-tail junction to the end of tail was measured. B. The length from head-tail junction to the sharp bend.

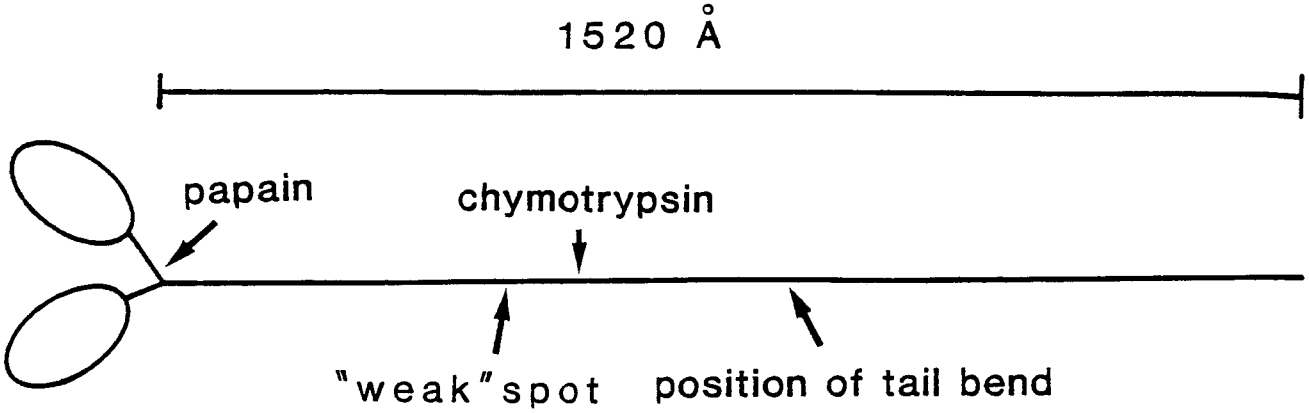

Fig. 10. Location of flexible region in the tail of the myosin molecule. A "weak" spot indicates possible flexibility of the rod predicted from amino acid sequence of myosin. The positions of a sharp bend and a proteolytic susceptible region in the tail were determined by the histograms in Fig. 9.

A junction between HMM and LMM has been considered to be impotant for force generation of muscle since it was reported on skeletal muscle that a large area of rod between S-2-LMM junction is flexible (2,3) and susceptible against protease (21). The rod sequence of unc-54 has features typical of an α-helical coiled coil, i.e., the hydrophobic amino acids show the expected seven-residue pattern, which modulated by a longer repeat of 28-residue zones (9). It is suggested that there is a "weak" point in which hydrophobic residues on the surface of the coiled coil disappears, and it is expected that a sharp bent and a cleavage point in rod might be observed only at this spot. The results presented here are contradictory to this idea. The molecular weight of HMM estimated from amino acid sequence is 132,068 daltons, but the molecular weight of HMM heavy chain measured from SDS-PAGE (150,000) was higher than this value (Figs. 2, & 4). HMM molecule resembles the myosin molecule with two globular heads attached to a tail, and the length of tail was about 55 nm (Fig. 9). However, the length from head to the "weak" spot (48 nm) is less than this value. Furthermore, the distance from head-tail junction to the sharp bend in the tail is much larger than these values (see Fig. 10). These results can be explained if -helical coiled coil structure of rod may be disrupted not only at the "weak" spot but within widely spreaded region. It is reported that large part in S-2 undergoes helix-coil melting with multiple local melting sites, and this part is considered to be an elastic component of the crossbridge (8). Furthermore, the above results and that the tail length of myosin observed by electron microscopy is 10 nm short of the

length estimated from amino acid support this notion.

The results presented here showed that the submolecular structure of myosin molecule is highly conserved between nematode and rabbit, though comparison between the unc-54 sequence (9,10) and known sequence fragment of rabbit skeletal myosin (11,12) shows only 52 % matching residues. These submolecular structure may be important for generation of muscle contraction.

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Part 3

Formation of Long Thick Filament Observed in Obliquely Striated Muscle by Unc-54 Myosin of the Nematode Caenorhabditis elegans

SUMMARY

The nematode Caenorhabditis elegans has obliquely striated muscle which has very long thich filaments (about 10 μm). We have purified myosin from the nematode. This myosin is mainly composed of myosin encoded by unc-54 gene and freed from paramyosin. Purified myosin aggregated into filaments at ionic strength below 0.13 M. The structure of filaments thus formed were observed by electron microscopy or by fluorescence microscopy after labelling with rhodamine isothiocyanate. We found that purified myosin formed a long filament (6-13 μm) even when paramyosin was absent. The length of myosin filament was similar to that of the thick filament of obliquely striated muscle observed in the body wall muscle.

INTRODUCTION

Muscle contraction occurs by the sliding of thick filament which is mainly composed with myosin past the thin filament which is mainly composed of actin (1,2). In the case of skeletal muscle the thick filament has 1.6 nm in length. This structure is formed by copolimerization of myosin itself (3-5). Nematode has obliquely striated muscle in which thick and thin filament are very long (6). Many kinds of invertebrate muscle have long thick filaments, and these muscle contains paramyosin (7). Paramyosin was also found in the nematode Caenorhabditis elegans (8). and paramyosin was considered to form a back bone of the thick filament.

Body wall muscle of the nematode Caenorhabditis elegans has two kinds of myosin, myosin A and B (9). Miller et al (10) showed that myosin B which is encoded by unc-54 gene is localized in the polar region, while myosin A is localized in the central 1.8 um of 9.7 um long thick filament. Epstein et al. (11,12) proposed that polar region of thick filament has core structure covered with paramyosin sheath and myosin B attaches to the surfase. They also claimed that myosin B is not required for the construction of long thick filament.

Recently, we purified myosin which is rich in unc-54 mysin by the extraction of myosin at low ionic strength (13). We found that paramyosin is resolved even under the conditions where myosin forms a thick filament (13). In the present study. we observed the formation of thick filament by myosin itself. Nematode myosin which is rich in unc-54 formed a long thick

filament which is seen in body wall muscle. The present result suggested that myosin B itself determine the length of thick filament even in non-vertebrate obliquely streated muscle, and paramyosin has a function to support this structure.

EXPERIMENTAL PROCEDURE

Preparation of Nematode Myosin -- The wild-type strain of nematode Caenorhabditis elegans, N2, was grown on an agar as described previously (11). Myosin was prepared from the worm by the method of Tanii et el (11). The concentration of nematode myosin was estimated by the method of Bradford (19), using dried nematode myosin as a standard. The SDS polyacrylamide slab gel electrophoresis (SDS-PAGE) (12.5 % acrylamide) was performed using buffer system of Laemmli (15). Gels were stained with Coomassie brilliant blue. The molecular weight of myosin was taken as 4.8×10^5.

Filament Formation-Myosin (final 0.2 mg/ml) was diluted into the buffer containing various concentration of NaCl, 2 or 10 mM $MgCl_2$, and 20 mM Imidazole-HCl at pH 7.0 and 0°C, and the light-scattering intensity at 350 nm was measured using Hitachi MPF-2 fluorescence spectrophotometer. The sample was also centrifuged at 10^4 x g for 10 min and the amount of protein in the supernatant was measured by the Bradford methods (19).

Electron micrograph of nematode myosin filament-Myosin (0.5 mg/ml) was dialyzed against the buffer containing 50 mM NaCl, 10 mM $MgCl_2$, and 20 mM Imidazole-HCl at pH 7.0 and 0°C, and then

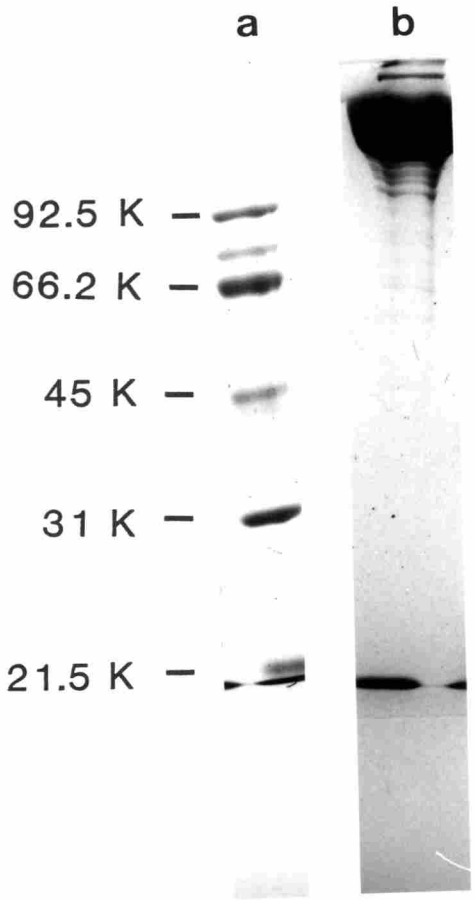

Fig. 1. SDS-gel electropholetic pattarn of nematode myosin. 10 % polyacrylamide-SDS gel. a, marker protein; b, 1 µg of purified myosin.

diluted to 0.05 mg/ml with the same buffer. A drop of suspension was placed on Formvar-carbon coated grid, treated with 2% uranyl acetate in distiled water and dried. Elecron microscopy was performed on a JEOL 100S operating ay 80 kv.

Fluorescence micrograph of nematode myosin filament- Myosin (0.7 mg/ml) was mixed with 7 μM rhodamine-isothiocyanide in the elusion buffer from DEAE-cellulose column (0.18 M NaCl, 1 mM $MgCl_2$, 20 mM imidazole-HCl, 0.2 mM ATP at pH 7.0 and 0°C), and stayed over night. Then, myosin was diluted into 0.02 μM with the buffer containing 50 mM NaCl, 10 mM $MgCl_2$, and 10 mM imidazole—HCl at pH 7.0 and 0°C. The fluorescence image of thick filament was photographed using a Olympus BHS-BFK fluorescence microscope.

RESULTS

Formation of Thick Filament by Unc-54 Myosin-Figure 1 showed a 12.5% polyacrylamide-SDS gelelectrophoretogram of myosin prepared from the nematode Caenorhabditis elegans. The major band of myosin fraction is the 210 K myosin heavy chain and the contamination of paramyosin and F-actin into the preparation were negligeble. The paramyosin band in the crude preparation was disappeared during the precipitation-dissolvement cycle in the preparation.

Figure 2 showed the dependence on NaCl concentration of the formation of thick filament measured by a centrifugation method. In 10 mM $MgCl_2$ and at 0.2 mg/ml nematode myosin about 75 % of myosin was precipitated at 0-20 mM NaCl by the centrifugation at

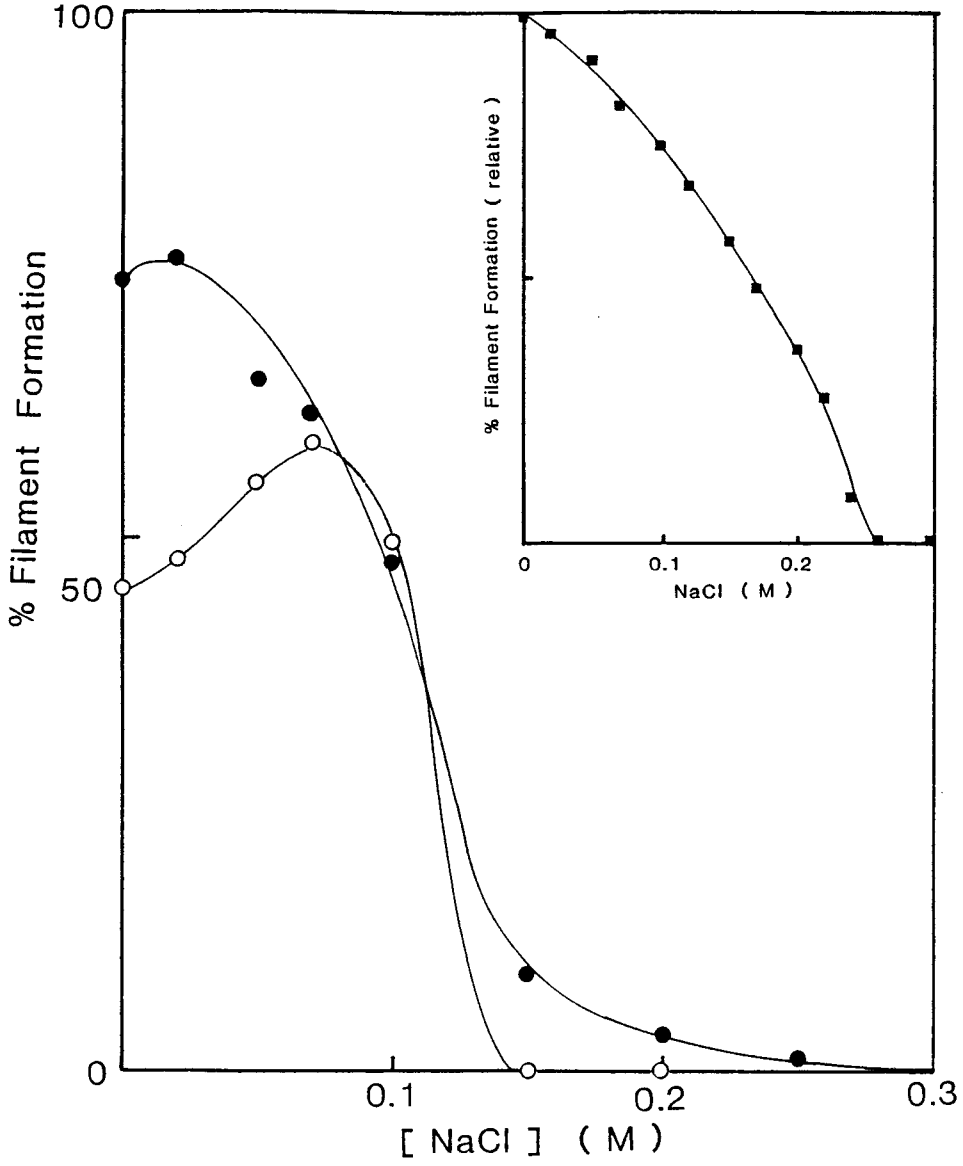

Fig. 2. Dependence on NaCl concentration of the formation of thick filaments. Myosin (final 0.2 mg/ml) was diluted into the buffer containing various concentration of NaCl, 2 (○) or 10mM (●) $MgCl_2$, and 20 mM Imidazole-HCl at pH 7.0 and 0 °C, and then centrifuged at 10^4 x g for 10 min. The amount of protein precipitated was estimated from the concentration of protein in the supernatant. The inserted figure shows the dependence on NaCl concentration of the light-scattering intensity at 350 nm. The conditions were the same except that the temperature was at 20 °C.

10^4 x g for 10 min. The amount of protein precipitated was decreased by increasing NaCl concentration above 0.1 M and myosin was dissoved almost completely at 0.25 M. When $MgCl_2$ concentration was decreased to 2 mM, the amount of protein precipitated at 0-20 mM NaCl was about 45 %. It incresed to about 60 % with increase in NaCl concentration to 0.075 M, then dissolved with increse in NaCl concentration to 0.15 M. The inserted figure in Fig. 2 shows the dependence on NaCl concentration of the light-scattering intensity at 350 nm of the solution containing 0.2 mg/ml nematode myosin and 10 mM $MgCl_2$ at 20°C. The light-scattering intensity decreased gradually with increase in NaCl concentration and reached to almost zero at 0.25 M NaCl.

Structure of Thick Filament Formed by Nematode Myosin-Myosin filaments formed in 10 mM $MgCl_2$, 50 mM NaCl and 20 mM Imidazole-HCl at pH 7.0 and 0°C were examined by electron microscopy after nagative staining. As shown in Fig. 3, thick filament with length of 6-13 μm were observed. The filaments have a diameter of around 15 nm and was decolated by projections with a diameter of about 10 nm. Very frequently, myosin filaments formed a side by side dimer with a diameter of 18-28 nm.

Figure 4 shows a fluorescence micrograph of myosin filament formed by myosin labelled with rhodamineisothiocyanate. The straight filaments with length of 8-15 μm were observed. We cannot determine the diameter of filament by fluorescence microscopy, and it is uncertain whether these filaments were dimer or not. However, we did not observe filaments with

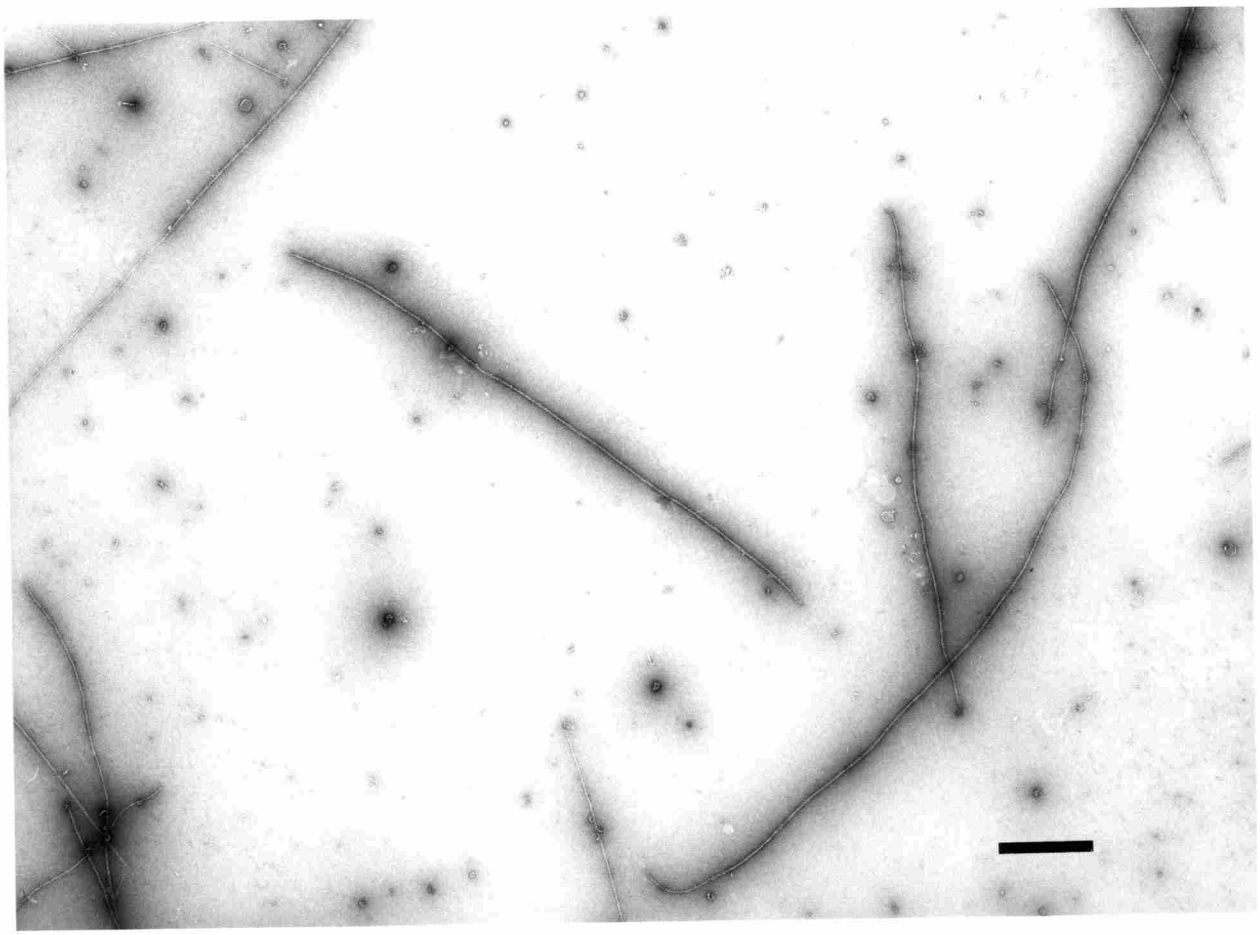

Fig. 3. Electron micrograph of nematode myosin filament. Myosin (0.5 mg/ml) was dialyzed against the buffer containing 50 mM NaCl, 10 mM $MgCl_2$, and 20 mM Imidazole-HCl at pH 7.0 and 0°C, and then diluted to 0.05 mg/ml with the same buffer. The sample was observed by electron micrography after nagative staining. magnification, x12670. Scale bar shows 1 μm.

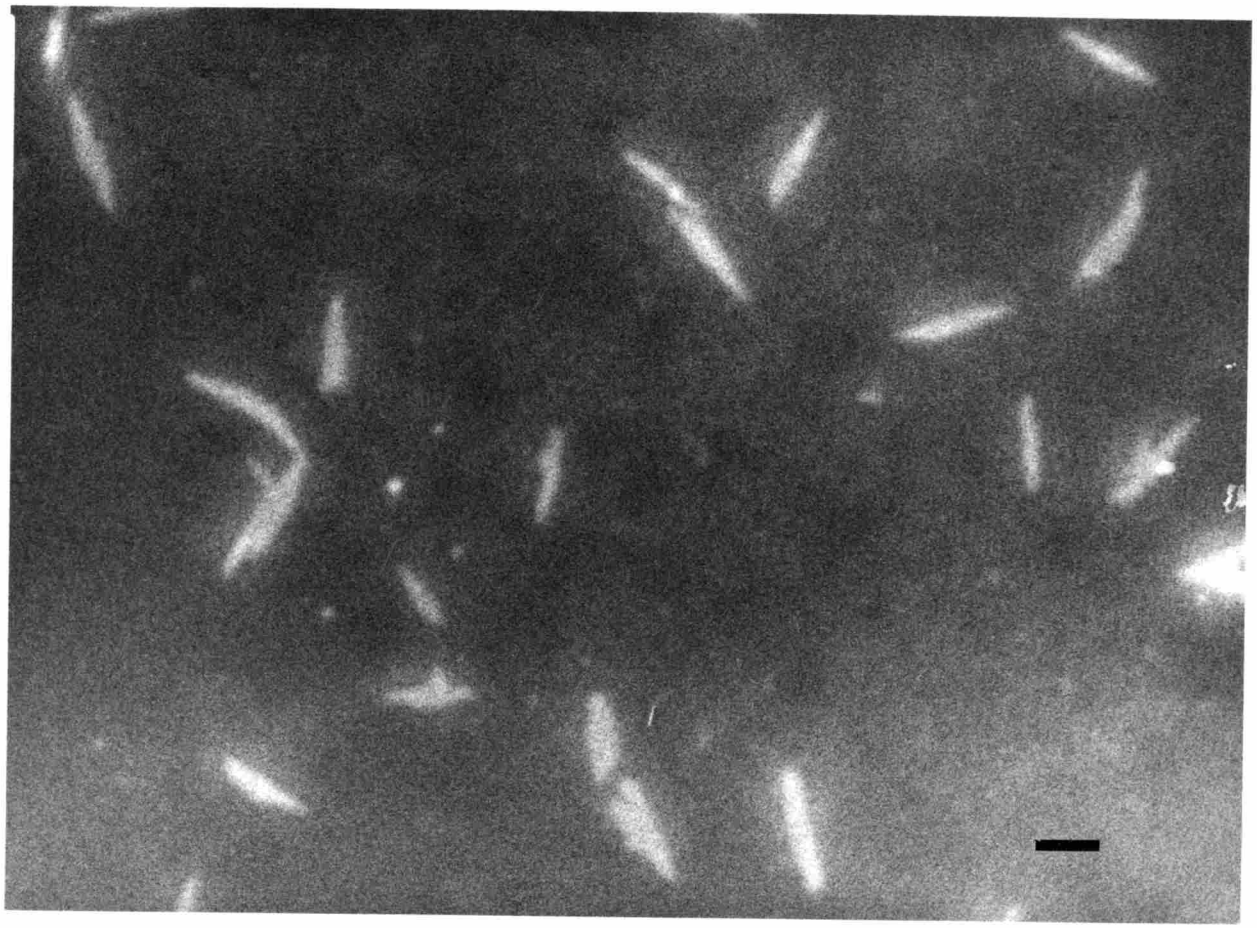

Fig. 4. Fluorescence micrograph of nematode myosin filament. Myosin (0.7 mg/ml) was modified with 7 μM rhodamine-isothiocyanide in 0.18 M NaCl, 1 mM $MgCl_2$, 20 mM imidazole-HCl, 0.2 mM ATP at pH 7.0 and 0 °C over night. Myosin was diluted to 0.02 uM with the buffer containing 50 mM NaCl, 10 mM $MgCl_2$, and 10 mM imidazole-HCl at pH 7.0 and 0 °C, and then observed by fluorescence micrograph. Magnification, x1750. Scale bar shows 5 μm.

different fluorescence intensity.

DISCUSSION

Body wall muscle of the nematode Caenorhabditis elegans has long thick filaments with about 10 um in length (6). This muscle contains two kinds of myosin, myosin A and B (9), and Miller et al (10) showed that myosin B which is encoded by unc-54 gene is localized in the polar region, while myosin A is localized in the center of the filament.

It was proposed by Epstein et al (11,12) that polar region of thick filament is formed by the three protein structure; central core, paramyosin sheath and myosin B in the surface of the filament, and that myosin B is not required for formation of long thick filament. We examined this possibility by studying the filament formation by the purified myosin. The results obtained here (Figs, 3 and 4) suggests that long thick filament is produced by myosin itself not by the other facters. Myosin itself aggregated at ionic strength around 0.1 M KCl.

Epstein et al (11) showed that there is a core structure in the thick filament anf this core structure is not dissolved even under high ionic strength. However, no core protein has not been identified, and Rosenbluth (16) reported the existence of filament network int he obliquelly striated muscle of nematode.

It was reported by several workers (7,17) that molluscan smmooth muscle which has a catch mechanism contains large quantity of paramyosin. Cohen et al. (7) reported that

paramyosin forms a core of thick filament and myosin attached the surfce of paramyosin core. The myosin of molluscan smooth muscle itself forms a small filament as skeletal muscle myosin (17,18). However, molluscan smooth muscle thin filaments is fuge; 150 nm in diameter and 30 um in length (19,20) which is much higher than the nematode thick filament.

We observed in the previous paper (13) that paramyosin is more soluble than myosin, and myosin can form a filament at almost physiological conditions (Fig. 2). Therefore three layer structure is not stable. Since paramyosin can attach to the thick filament and paramyosin deficient mutant of C. elegans has smaller thich filament (12), paramyosin may support the structure required for the construction of long thick filament.

Recently, we purified myosin which is rich in unc-54 mysin by the extraction of myosin at low ionic strength (13). We found that paramyosin is resolved even under the conditions where myosin forms a thick filament (13). In the present study. we observed the formation of thick filament by myosin itself. Nematode myosin which is rich in unc-54 formed a long thick filament which is seen in body wall muscle. The present result suggested that myosin B itself determine the length of thick filament even in non-vertebrate obliquely streated muscle, and paramyosin has a function to support this structure.

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Part 4

Purification of Unc-54 Myosin from E675 Mutant of Nematode Caenorhabditis elegans and the Heterogeniety in the Two Heads of Myosin

(SUMMARY)

A soil nematode, Caenorhabditis elegans produces four kinds of myosin heavy chain isoforms. The E675 mutant, one of the alleles of unc-54 myosin heavy chain gene produced a unique molecular form of myosin heavy chain of about 203.000 molecular weight in addition to normal 210.000 molecular weight heavy chain. The myosin with 203.000 dalton heavy chain was purified from E675 mutant. The SDS-polyacrylamide gel electrophoresis pattern of myosin from E675 mutant showed that most of myosin heavy chain (94%) in the preparation was encoded by the unc-54 gene. This myosin has ATPase activity and F-actin binding ability as wild type myosin. The myosin bound with 1.7 mol of nucleotide per mol of myosin. However, the amount of myosin-phosphate-ADP complex, M_P^{ADP}, measured from the size of Pi-burst was 1 mol per mol of myosin. Therefore, one of the two heads forms M_P^{ADP}. One mol of specific lysine residue per mol of myosin was rapidly trinitrophenylated when myosin was modified with 2,4,6-trinitro benzenesulfonate (TNBS), and further one mol of lysine residue was modified slowly with TNBS. The EDTA(K^+)-ATPase activity which is catalyzed by M_P^{ADP} site decreased coincidentally with the rapid trinitrophenylation. Therefore, we concluded that only the specific lysine residue in M_P^{ADP} forming head is rapidly modified with TNBS. These results suggested that myosin encoded by unc-54 gene has non-identical heads as mammalian skeletal muscle myosin has though both heads are encoded from a single gene.

INTRODUCTION

Myosin has two separated heads which can bind with F-actin and reacts with ATP. The structures and functions of these two heads have been studied mainly using rabbit skeletal muscle myosin. We (1) showed that rabbit skeletal muscle myosin forms different ATPase reaction intermediate, i.e., the myosin-phosphate-ADP complex is formed by one of the two heads of myosin (head B or burst head) and the other head (hea A or nonburst head forms the myosin-ATP complex. The actomyosin ATPase rection which is coupled with muscle contraction was catalyzed by head B while head A is considered to work for smooth movement of myosin heads along F-actin. Miyanishi et al. (2,3) showd that the chemical structure around the reactive lysine residue are different between heads B and A. According to them (3) a proline residue located near the reactive lysine residue in head B is replaced by a serine in head A. Thus, the difference in the function of two heads are considered to be derived from the different chemical structure between two heads. However, rabbit skeletal muscle contains several isoforms of the heavy chain (4-7), and the possibility that the difference in the chemical structure between heads B and A is due to the existence of these isoforms still remains. Then, it needs the myosin which contains single isoform to know wheather the two heads of myosin are structurary nonidentical or not.

A soil nematode Caenorhabditis elegans has has four myosin heavy chain (MHC) genes which encoded distinct myosin heavy chain from each other, and express in a tissue specific and

Table I. Myosin isoforms of the nematode *C. elegans*.

myosin	gene	Location	N2 (wild type)	E675 (mutant)
myosin D	*myo-1*	Pharynx	206 K	206 k
myosin C	*myo-2*	Pharynx	210 K	210 K
myosin A	*myo-3*	Body Wall	210 K	210 K
myosin B	*unc-54*	Body Wall	210 K	203 K

developementally regulated manner (Table I)(8,9). In the previous paper (10) we described the preparation of unc-54 myosin which is expressed in body wall muscle. However, the contamination of other myosin isoforms can not be excluded. In the present paper we prepared myosin from a mutant E675, one of the alleles of unc-54 by the method described previously (10). In this mutant the size of MHC encoded by unc-54 has 203.000 molecular weight which is resulted from deletion in tail part of the myosin molecule (11,12). The SDS-PAGE pattern of myosin prepared from E675 contained mainly the 203.000 molecular weight MHC. Thus, the myosin encoded from sigle myosin heavy chain gene was obtained. In the present paper we intended to examine whether the two heads of myosin are identical or not. The results obtained showed that two heads of myosin are nonidentical eve though they are produced from a single gene.

EXPERIMENTAL PROCEDURE

Materials-N2, the wild type strain of Caenorhabditis elegans, and E675, the unc-54 mutant obtained by Brenner (11) using methanesulfonate, were grown using plate dishes as described previously (10,14). Myosins were prepared from N2 and E675 worms by the method described in the previous paper (10). Rabbit skeletal muscle myosin was prepared by the method of Perry (15). Actin was prepared from an acetone powder of rabbit skeletal muscle by the method of Spudich and Watt (16). Protein concentrations were determined by the method of Bladford (17),

using dried proteins as standard. The molecular weight of E675 myosin adopted was 472.000.

ATP was purchased from Kohjin Co., LTD. (γ-^{32}P)ATP was synthesized enzymatically by the method of Schendell and Wells (18). (γ-^{32}P)ATP was purchased from Radiochemical Center Amersham, England. (α-^{32}P)ADP was obtained by enzymatic hydrolysis of (α-^{32}P)ATP using myosin and was purified according to the method of Miyata (19).

Methods-SDS-PAGE (4.5 % polyacrylamide) was performed using the buffer system of Laemmli (20). Gels were stained with Coomassie Brilliant Blue.

The ATPase reaction of myosin was measured from the time course of ^{32}Pi-liberation using (γ-^{32}P)ATP as substrate. The reaction medium usually contained 0.5 M NaCl, 10 mM $MgCl_2$, 0.1 mM EDTA, 50 mM Imidazole-HCl at pH 7.0 and 0°C. The reaction was started by mixing 0.35 µM myosin with 5 µM (γ-^{32}P)ATP and the reaction was quenched with 55%TCA and 0.1 mM cold Pi as carrier. The amount of ^{32}Pi was determined as described previously (21). The EDTA(K^+)-ATPase activity was measured from the steady-state rate of ATP hydrolysis. The amounts of Pi liberated was measured by the methods o f Youngberg and Youngberg (22).

The amount of ADP bound to myosin in the presence of Vi ions (23) was determined as follows. Various concentrations of (α-^{32}P)ADP was incubated with 0.5 µM E675 myosin in 0.5 M KCl, 2 mM $MgCl_2$, 0.1 mM NaVi, 0.1 mM EDTA, and 20 mM Imidazole-HCl at pH 7.0 and 0°C for 3 h. The reaction was quenched with 2.5 mM each of cold ADP and cold ATP. After adding 0.25 mg/ml rabbit skeletal muscle myosin as carrier, The nematode myosin-Vi-ADP

complex was collected by ammonium sulfate fractionation (60%). After centrifugation at 10^4 x g for 20 min, the radioactivity in the presipitate was measured.

The binding of myosin with F-actin was measured by a centrifugation method. The mixture containing 1 μM myosin and 5 μM F-actin in the buffer was centifuged at 1.6 x 10^5 g for 1 hr. The amount of myosin and actin in the supernatant was measured from the staining density of bands after SDS-PAGE.

The time-course of trinitrophenylation of myosin by TNBS was followed as described by Miyanishi et al (2). E675 myosin (0.84 uM) was allowed to react with 0.25 mM TNBS in 0.5 M KCl, 0.2 mM EDTA, and 20 mM Imidazole-HCl at pH 7.0 and 20 °C. The amount of TNP bound to myosin was monitored from the increase in OD at 345 nm using 1.45 x 10^4 as the molar extinction coefficient of trinitrophenyl group bound to ε-amino group of protein (2).

RESULTS

Selective Purification of Unc-54 Myosin-Myosins were prepared from wild type nematode, N2, and unc-54 mutant, E675, as described previously (10) using low salt extraction and DEAE-cellulose column chromatography. Figure 1 shows the 4.5% SDS polyacrylamide gel electrophoresis (PAGE) pattern of myosin purified from N2 (lane a) and E675 (lane b). The molecular weight of myosin heavy chain (MHC) from E675 was 203.000, while that from N2 210.000. The area of 203.000 dalton MHC was 94 % oftotal MHC area.

The ATPase activities of myosins from E675 and N2 were

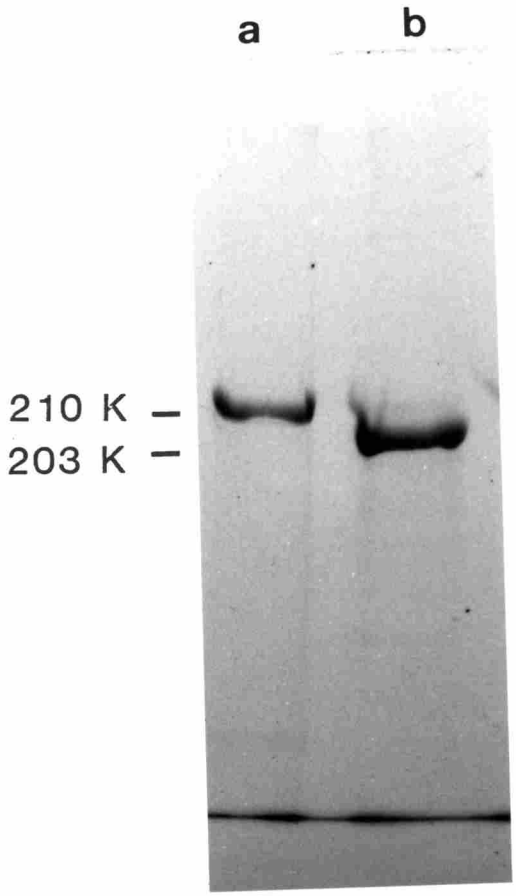

Fig. 1. SDS-gel electrophoresis pattern of nematode myosin. 4.5 % acrylamide gel. (a) 1 µg myosin of wild type namatode. (b) 2 µg E675 myosin.

Table II. ATPase activity of C. elegans myosin at the steady state. The assay mixture contained 50 mM imidazole-HCl at pH 7.0 The EDTA(K^+)-ATPase activity was measured in 0.7 mg/ml myosin, 10 mM EDTA, 1 mM ATP and 0.5 M KCl at 20°C. The Mg^{2+}-ATPase activity was measured in 0.16 mg/ml myosin, 10 mM $MgCl_2$, 5 μM ATP, 0.5 M NaCl at 0°C.

	N2 myosin (s^{-1})	E675 myosin (s^{-1})
EDTA(K^+)-ATPase	2.29	1.53
Mg^{2+}-ATPase	0.012	0.021

measured in the presence of EDTA or Mg^{2+}, and the results were shown in Table II. The EDTA(K^+)-ATPase activity and the Mg^{2+} ATPase activity of E675 myosin were 1.53 and 0.021 s^{-1}, respectively, which were almost equal to those of N2 (2.29 and 0.012 s^{-1}, respectively).

The binding of E675 myosin with F-actin (rabbit skeletal muscle) was studied by a centrifugal separation method. The mixture (0.5 ml) of 0.3 mg/ml myosin from E675 and 0.77 mg/ml F-actin in 0.5 M NaCl, 2 mM $MgCl_2$, and 20 mM imidazole-HCl at pH 7.0 and 0 °C was centrifuged at 10^5 x g for 1 hr. The supernatant was examined by SDS-PAGE (see Table III). The mixture was also centrifuged in the presence of 5 mM ATP. The amounts of F-actin precipitated in the absence and presence of ATP were 74.4% and 88%, respectively. Most of myosin (73%) was precipitated in the absence of ATP, while myosin was not precipitated in the presence of 5 mM ATP.

Pi-Burst Size of Unc-54 Myosin-The amount of myosin-phosphate-ADP complex formed by unc-54 myosin was studied from the size of initial burst of Pi-liberation using ^{32}P ATP as substrate. Figure 2 shows the time course of ^{32}Pi liberation whe 2 μM ^{32}P ATP was mixed with 0.35 μM E675 myosin in 10 mM $MgCl_2$, 0.5 M NaCl and 50 mM imidazole-HCl at pH 7.0 and 0 °C. About 1 mol of ^{32}Pi per mol of myosin was liberated rapidly in the initial phase of the reaction, and then liberated slowly at the steady state rate of 0.021 s^{-1}. The size of the Pi-burst obtained by extapolating the time course of Pi-liberationto time zero was 1.04 mol per mol of myosin.

Amount of Nucleotide Bound to Myosin-We measured the

Table. III. Binding of nematode E675 myosin with F-actin in the presence and absence of ATP. A mixture (0.2 ml) of 0.3 mg/ml myosin and 0.77 mg/ml F-actin in 0.5 M NaCl, 2 mM $MgCl_2$, and 20 mM imidazole-HCl at pH 7.0 and 0°C was centrifuged at 1.6 x 10^5 g for 1 h in the presence and absence of 5 mM ATP. The supernatant was examined by SDS-PAGE. The amount of myosin and actin were determined from the band intensities after staining the gel.

	Myosin (mg/ml)	Actin (mg/ml)
Before centrifugation	0.30 (100)	0.77 (100)
Supernatant (-ATP)	0.08 (26.7)	0.20 (25.6)
Supernatant (+ 5 mM ATP)	0.28 (94.3)	0.09 (12.0)

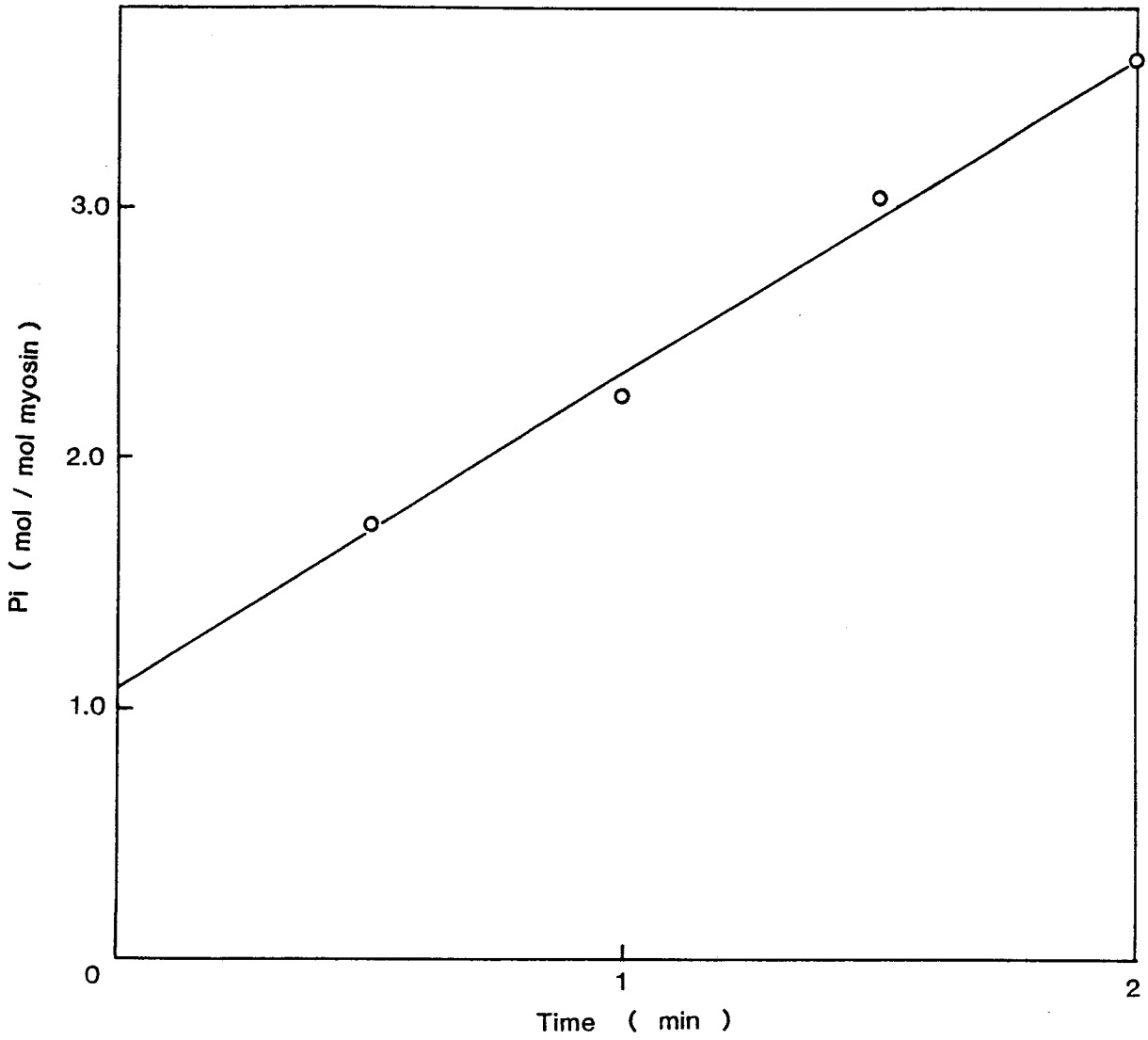

Fig. 2. Time course of Pi liberation in the initial phase of nematode E675 myosin. The reaction medium contained 0.35 μM myosin, 0.5 M NaCl, 10 mM $MgCl_2$, 20 mM imidazole-HCl at pH 7.0 and 0 °C. The reaction was started by adding 5 μM (γ-^{32}P)ATP, and stopped by adding 10% TCA containing 0.1 mM Pi as carrier.

amount of ADP bound to myosin in the presence of Vi ions to know whether two heads of myosin are active or not. It was shown by Goodno (21) that skeletal muscle myosin forms stable myosin-Vi-ADP complex in the presence of Vi ions. Then, 2 μM of nematode myosin was incubated with various concentration of α-^{32}P ADP in the presence of 1 mM NaVi, in 0.5 M KCl, 10 mM $MgCl_2$, and 20 mM Imidazole-HCl at pH 7.0 and 0 °C for 5 hr. Figure 3 shows the amount of nucleotide bound to myosin as a function of α-^{32}P ADP added. The amount of myosin-V-ADP complex increased with increase in ADP concentration and reched the maximal level of about 1.7 mol per mol of myosin when 10 μM α-^{32}P ADP was added to myosin.

Trinitrophenylation of Unc-54 Myosin-We (2) have shown in skeletal muscle myosin that myosin has 2 mol of reactive Lys residues per mol of myosin and reactivity of 1 mol of Lys in head A was decreased in the presence of ADP or PPi. Then, we measured the time course of trinitrophenylation of myosin after adding 0.25 mM TNBS to 3.5 μM E675 myosin (Fig. 4). Trinitrophenylation occured by three phases; an initial rapid phase was followed by a second slower phase and then followed by a third slower phase. The amount of TNP bound in the rapid phase and in the secon phase were each one mol per mol of myosin, respectively. When 1 mM ADP was added during trinitrophenylation the rate of first rapid phase and that of second phase was decreased to about one third that in the absence of ADP, but the amounts of TNP bound in the first and the second phase were unaffected (data not shown).

Figure 5 showed the effect of trinitrophenylation on the EDTA(K^+)-ATPase activity of E675 myosin. The EDTA(K^+)-ATPase

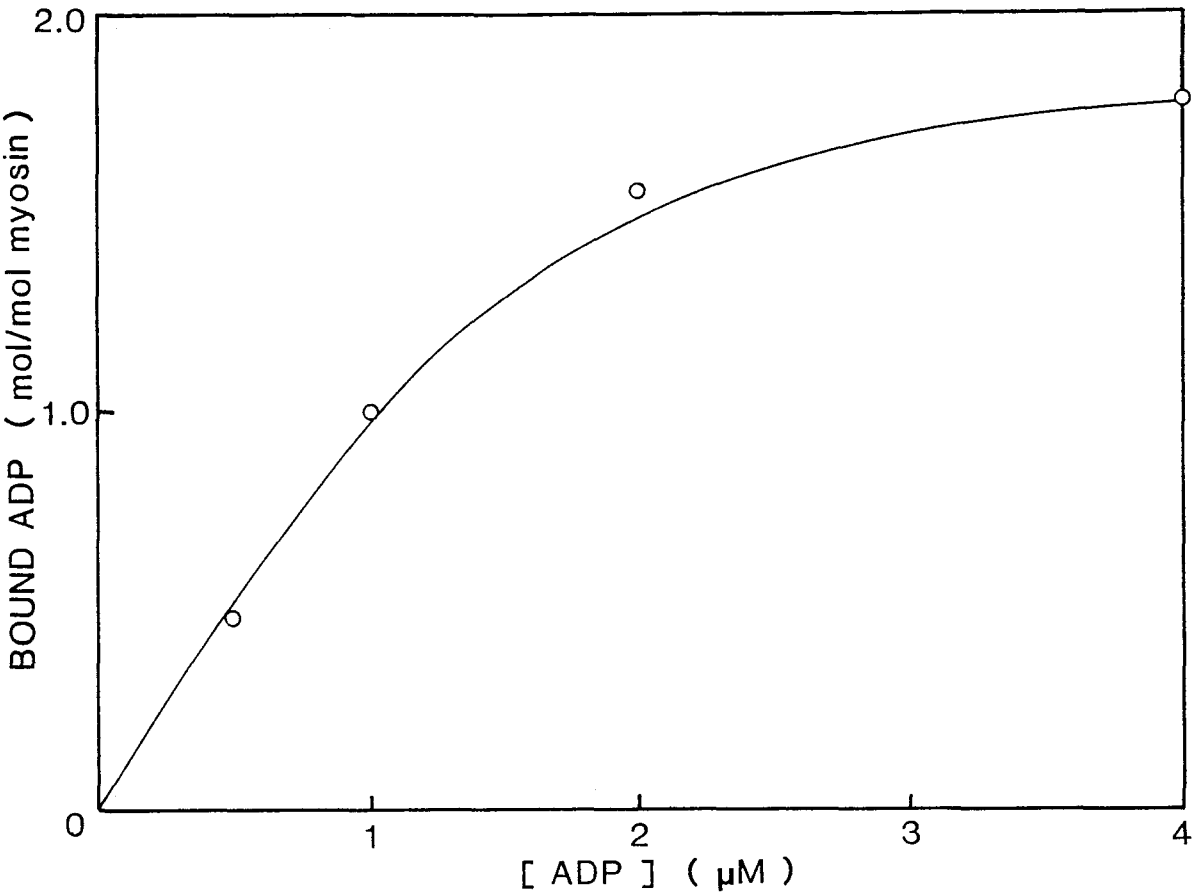

Fig. 3. Amount of ADP bound to nematode E675 myosin in the presence of vanadate. Various concentration of (α-^{32}P)ADP was incubated with 0.5 μM E675 myosin in 0.5 M KCl, 2 mM $MgCl_2$, 0.1 mM NaVi, 0.1 mM EDTA and 20 mM imidazole-HCl at pH 7.0 and 0°C for 3 h.

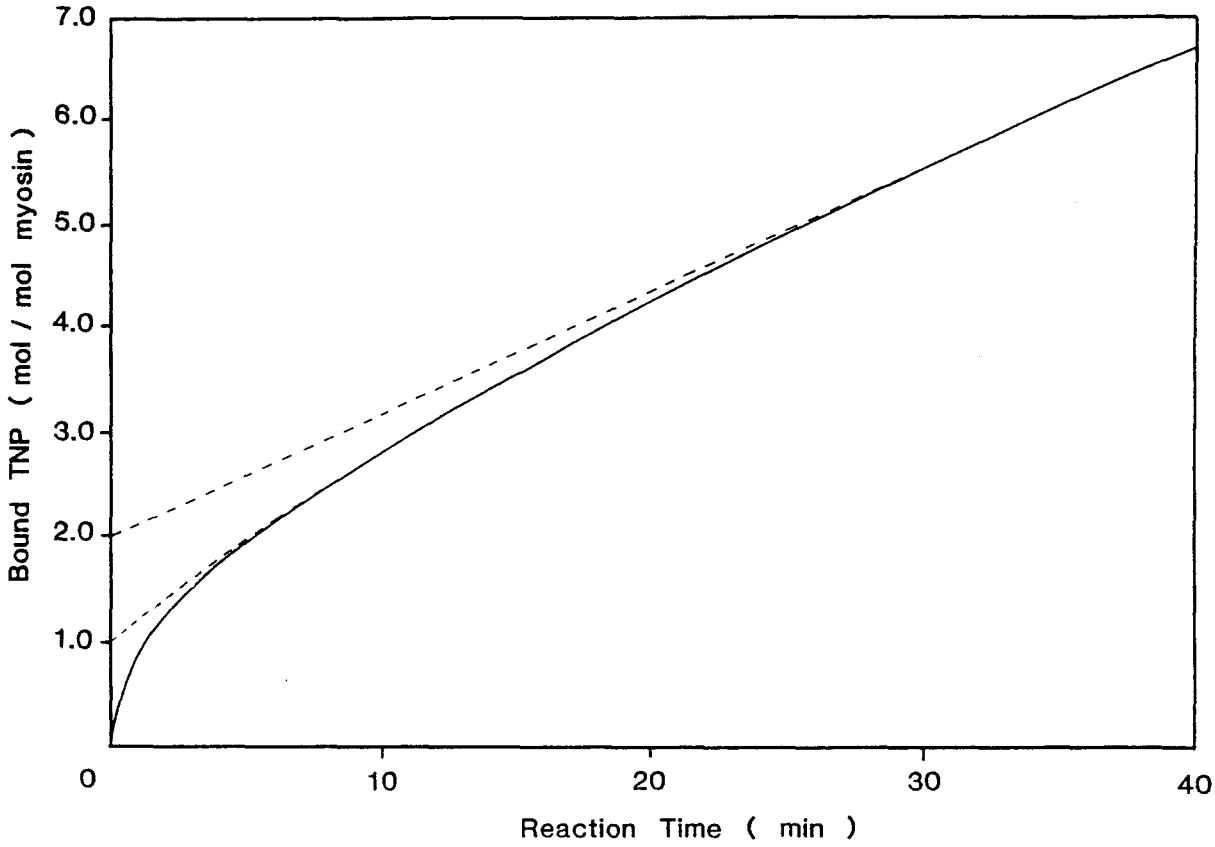

Fig. 4. Time course of trinitrophenylation of E675 myosin E675 myosin (0.84 μM) was allowed to react with 0.25 mM TNBS in 0.5 M KCl, 0.2 mM EDTA, and 20 mM imidazole-HCl at pH 7.0 and 20°C.

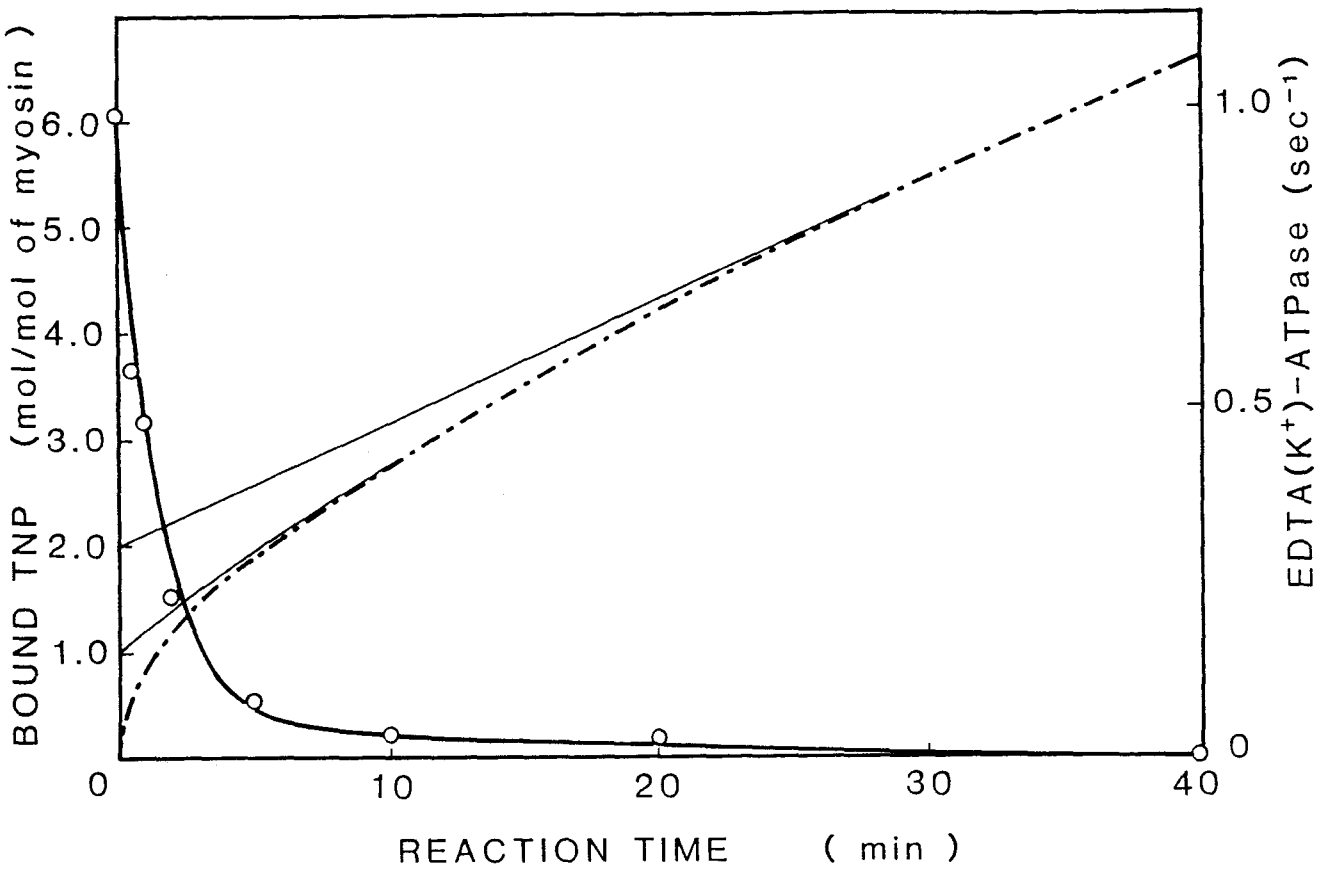

Fig. 5. Effect of trinitrophenylation on the EDTA(K^+)-ATPase activity of E675 myosin. Myosin was reacted as described for Fig. 4. The reaction was stopped by adding 20 mM DTT. After removing free TNBS and DTT by centrifuge column, EDTA(K^+)-ATPase was assayed in 0.1 uM myosin 0.5 M KCl, 10 mM EDTA, 2 mM ATP and 20 mM imidazole-HCl at pH 7.0 and 20°C.

activity decresed rapidly from 2.2 s^{-1} to 0.46 and 0.33 s^{-1} at 2 and 5 min.

DISCUSSION

The nematode Caenorhabditis elegans has four myosin heavy chain genes each of which encodes single distinctive myosin heavy chain (MHC)(ref.8, Table I). Over 100 mutations in the unc-54 have been isolated during the course of genetic analysis of muscle defective strains; 30 of these mutations have been ordered on a genetic fine structure map (24,25). In the present paper, we used the mutant E675, one of the alleles of the unc-54. The mutation of E675 is an in-phase deletion of 270 bp, which result in a shortened myosin rod segment (13). The size of MHC produced by the unc-54 of E675 is 203.000 MW, which is distinguishable from other three MHC (see Table I).

In the previous paper (10), we prepared myosin from nematode by extraction at low ionic strength. The unc-54 myosin was selectively extracted in this condition since the solubility of unc-54 myosin was higher than the other type of myosins. In the present paper we prepared unc-54 myosin by the same way as that from N2. The unc-54 myosin from E675 strain dissolved at slightly lower ionic strength than myosin from N2 (Tanii et al. to be submitted). The SDS-PAGE pattern showed that about 94 % of total MHC was encoded by the depleted unc-54 gene (Fig. 1).

The ATPase activity and the F-actin binding ability of unc-54 myosin from E675 were the same as those of wild type myosin (Fig. 2, Table II). These correspond to the genetic analysis that the

mutation in E675 is not located in the head but within the rod portion of the molecule.

Since unc-54 myosin from E675 was encoded from a single gene, we examined whether the structure and function of two heads of this myosin are identical or not. In rabbit skeletal muscle myosin the two heads were shown to be non-identical (1,2). However, there are many isoforms of myosin in rabbit (4-7), and the results can be interpreted by the isoforms. The size of initial burst of Pi-liberation was 1 mol per mol of myosin (Fig.2), though myosin can bind with 1.7 mole of nucleotides per mol of myosin (Fig. 3). These results indicate that the myosin-phosphare-ADP complex, M_P^{ADP}, is formed in one of the two heads of myosin.

Skeletal muscle myosin has two reactive lysine residue in each heads of the molecule, and the reactivity of the lysine in the head A is suppressed by ADP or PPi. Later, we found that the amino acid sequence around the reactive lysine residue are different between heads B and A. The unc-54 myosin has also two reactive lysine residues, one of which reacts more rapidly than the other (Fig. 4). The EDTA(K^+)-ATPase activity, which is catalyzed by head B decreased accopanying the rapid phase of trinitrophenylation (Fig. 5). Therefore, we concluded that rapidly trinitrophenylated lysine is located on head B. In the case of rabbit skeletal muscle, the observed size of Pi-burst decreased by the trinitrophenylation of head B, however the Pi-burst of nematode myosin was not disappeaared by the trinitrophenylation of myosin and the rate of trinitrophenylation in head B was decreased by the presence of ADP. These results

suggest that the reactive lysine residue is not located in the active site of ATPase.

It is considered from the analogy of rabbit skeletal muscle myosin that the primary structure around the reactive lysine residue of two heads of nematode myosin are also different from each other, and it may result the difference in the function of two heads. However, it was shown from the RNA blot hybridization analysis that the size of mRNA produced by unc-54 gene is only for one MHC. It was reported on the rabbit skeletal muscle myosin that there are microhetrogeniety in the amino acid sequence (4-6). These microheterogeniety are difficult to explain by the splicing mechanism. Whether different mRNA with same size are produced by a specific mechanism or different proteins are synthesized from a same mRNA will be examined in this animal.

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Part 5

Regulation of Actomyosin ATPase Reaction of Nematode *Caenorhabditis elegans*

SUMMARY

The regulatory system of body wall muscle of nematode Caenorhaditis elegans was studied by measuring the Mg^{2+}-ATPase activity of actomyosin in the presence or absence of Ca^{2+}ions. Nematode actin fraction, which contained actin, tropomyosin and other minor protains, increased the Mg^{2+}-ATPase of rabbit skeletal myosin in a Ca^{2+}-dependent manner. Actin and other protains were separated by centrifugation in 0.5M KCL at 10^5 g for 3 hr. The nematode actin thus obtained precipitated increased the Mg^{2+}-ATPase activity of rabbit skeletal myosin in a Ca^{2+}-insensitive manner. The supernatant fraction made the ATPase activities of actin-myosin system into Ca^{2+}-dependent. On the other hand, the Mg^{2+}-ATPase of C. elegans myosin was activated by rabbit skeletal actin about 20 times in a Ca^{2+}-insensitive manner. The gizzaard native tropomyosin fraction which regulate the smooth muscle actomyosin ATPase by Ca^{2+}-dependent phosphorylation of myosin light chain caused slight enhancement of acto-nematode myosin ATPase activity, but did not induce the significant effect on the Ca^{2+} sensitivity of actomyosin ATPase. The results obtained suggest that nematode muscle is controled by the tropomyosin-troponin like proteins through the thin filament.

INTRODUCTION

The contractile system of nematode Caenorhabditis elegans has been well studied not only biochemically (1-4) but also genetically from the DNA level (5-8). However, the mechanism of regulation of contraction of this animal has not yet been studied. three kind of Ca^{2+}-regulatory system has been reported in animal kingdam. Contractions of all muscle which has been studied are regulated by the level of sarcoplasmic Ca^{2+}. However, the mechanism of Ca^{2+}-regulation depends on species of animal and muscle type. Manmarian skeletal muscle is regulated by tropomyosin-troponin system (9) which binds with thin filament and Ca^{2+} ions bind with troponin-C (10). On the other hand, types of myosin-linked regulation have been reported. One is based on the Ca^{2+}-dependent phosphorylation and dephosphorylation of the myosin light chain as in smooth muscle (11,12).The other is the direct binding of Ca^{2+} to myosin light chain as reported in scallope muscle (13).

Recentry we succeeded in preparing unc-54 rich myosin and actin from the nematode C.elegans using DEAE-cellulose column chromatography. We found that the mixture of purified myosin and actin fraction gave the Ca^{2+} sensitive actomyosin-type ATPase reaction. The actin fraction was separated into pure actin and regulatory proteins. By using these proteins, Ca^{2+}-regulatory system in nematode C.elegans was investigated. We concluded that actin-linked regulatory system, such as tropomyosin-troponin system exists in C.elegans and that there is no myosin-linked regulation in C.elegans actomyosin.

EXPERIMENTAL PROCEDURE

Nematode Strain and growth - The wildtype strain of nematode Caenorhabditis elegans, N2, which was initially isolated by S. Brenner (14), was kindly supplied by Dr.R.Hosono of Kanazawa University. The nematode was grown on an agar by procedure of Sassa et al.(15) with slight modification (2).

Preparation of Proteins - Nematode myosin and actin were prepared as described previously (2). Nematode actomyosin fraction was separated into myosin and actin fraction by DEAE-cellulose column chromatography. Myosin was eluted with the buffer containing 0.18 M NaCl, 0.2 mM ATP, 1 mM $MgCl_2$, 1 mM DTT, and 20 mM imidazole-HCl at pH 7.0. The actin fraction was eluted with the buffer containing 0.5 M KCl, 1 mM DTT, and 20 mM imidazole-HCl at pH 7.0 at a flow rate of 50 ml/h. Figure 1A and B show the SDS-PAGE (15%) patterns of the actomyosin fraction and the actin fraction, respectively. Actin fraction displayed the main bands of actin (42.5 K daltons), and tropomyosin (40 K daltons). The actin fraction was centrifuged at 10^5xg for 3 hr and the precipitate was suspended in the same buffer used for eluting actin. The recovery of proteins in the supernatant was about 60 % that in the actin fraction. Figure 1C and D show the SDS-PAGE patterns of the supernatant and the precipitate, respectively, which demonstrate that actin and tropomyosin were separated into distinct fractions. The proteins were dialyzed against the solution containing 50 mM KCl, 1 mM DTT, and 20 mM imidazole-HCl at pH 7.0 for the ATPase assay.

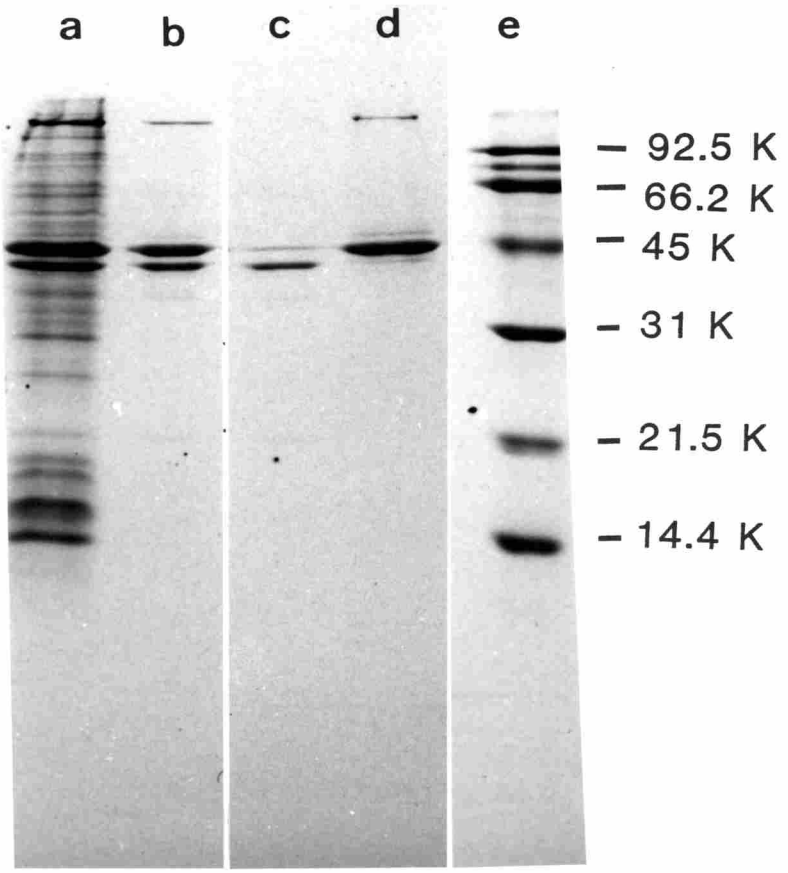

Figure 1. SDS-gel electrophoretic patterns of actin fraction, actin and the regulatory proteins. 15 % SDS-PAGE of (a) actomyosin fraction, (b) actin fraction, (c) supernatant, (d) precipitate after centrifugation of the actin fraction, (e) molecular weight markers, phosphorylase b (94 K), bovine serum albumine (67 K), ovalbumine (43 K), carbonic anhydrase (30 K), soybean trypsin inhibitor (20.1 K),α-lactalbumine (14.4 K).

Skeletal muscle myosin and actin were prepared from rabbit skeletal muscle by the methods of Perry (16), Spudich and Watt (17), respectively. Chicken gizzard native tropomyosin (NTM) was prepared by the method of Ebashi (18).

The concentrations of skeletal muscle myosin, skeletal muscle actin and gizzard native tropomyosin were estimated by the Biuret method, with calibration by nitrogen determination or dry weight. The concentrations of nematode myosin and actin fraction were estimated by the method of Bradford (19), using dried nematode myosin or dried actin fraction as a standard, respectively. The concentration of proteins in other fractions were also estimated by the Bradford method.

SDS Polyacrylamide Gel Electrophoresis - SDS polyacrylamide slab gel electrophoresis was performed using 15 % acrylamide gel in the buffer system of Laemmli (20). Gels were stained with Coomassie brilliant blue. Molecular weight markers were purchased from Sigma Chemical Co.LTD.

Actomyosin-ATPase Reaction - The ATPase reaction was carried out at 20°C in a medium containing 50 mM KCl or 20 mM KCl, 2 mM $MgCl_2$, 20 mM imidazole-HCl at pH 7.0, 1 mM $CaCl_2$ or 2.5 mM EGTA and an appropriate concentration of contractile proteins. The ATPase activity was determined by measuring the rate of Pi liberation according to the method of Youngburg and Youngburg (21).

RESULTS

The nematode myosin and the actin fractions were obtained by

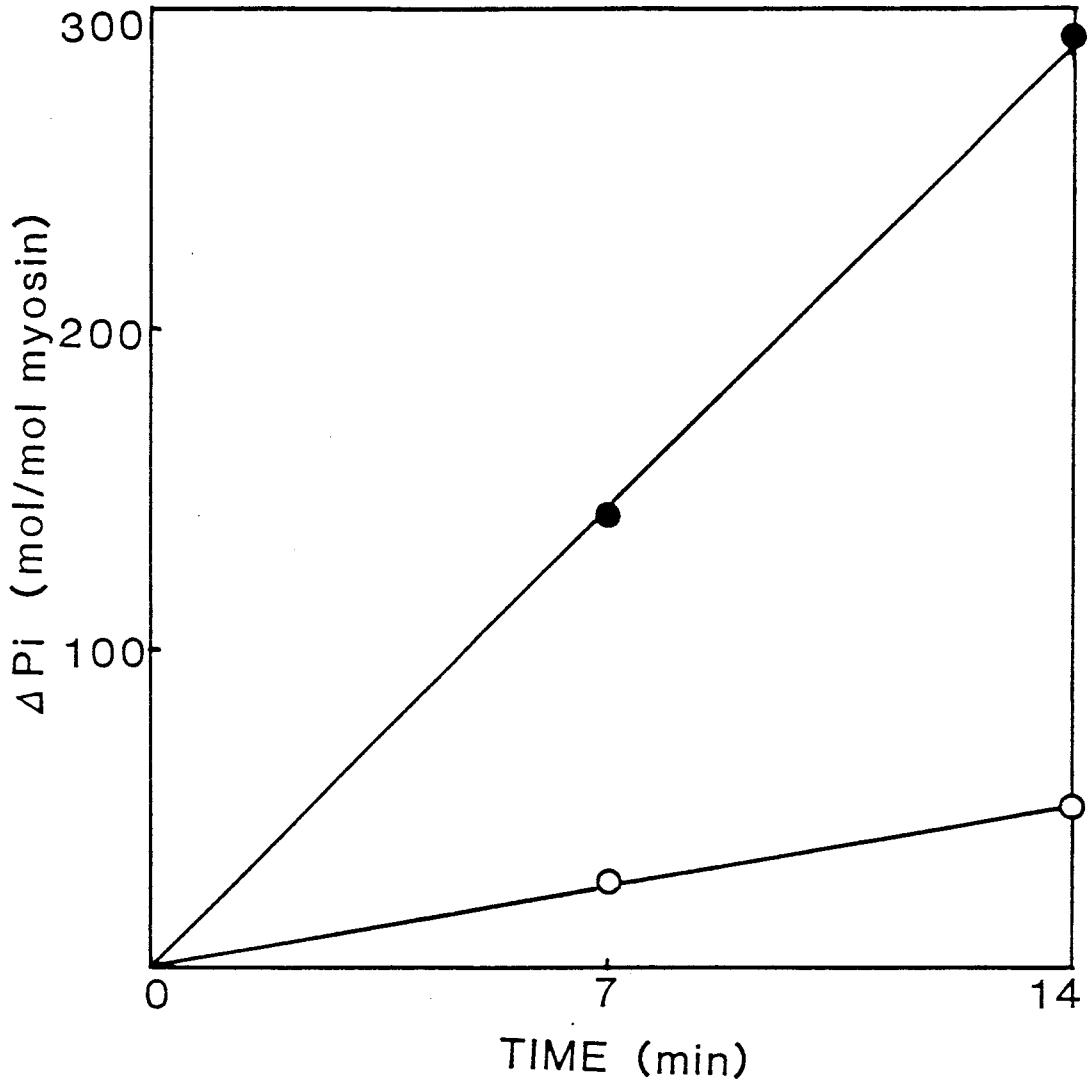

Figure 2. Ca^{2+}-control of actomyosin ATPase reaction reconstituted by skeletal myosin and nematode actin fraction. 0.2 mg/ml skeletal muscle myosin (0.42 μM), 0.17 mg/ml nematode actin fraction, 20 mMKCl, 0.5 mM ATP, 2 mM $MgCl_2$, 10 mM imidazole-HCl (pH 7.0) and 20 °C, 1mM $CaCl_2$ (●) or 5 mM EGTA (○).

TABLE I. Actin-linked Ca^{2+}-control of nematode actomyosin ATPase. ATPase activities were assayed in the solution (1.2 ml) containing 0.2 mg/ml rabbit skeletal myosin, 20 mM KCl, 2 mM $MgCl_2$, 0.5 mM ATP, 20 mM imidazole-HCl (pH 7.0) and 1 mM $CaCl_2$ or 2.5 mM EGTA at 20 °C, and when added, 0.05 mg/ml rabbit skeletal actin, 400 µl of actin fraction (final 0.1 mg/ml), 400 µl of the precipitate fraction, 400 µl of the supernatant fraction.

Condition	Activity (min^{-1})	
	+ Ca	+ EGTA
skeletal myosin	2.7	2.7
sk. myosin + nematode actin (original)	21.0	3.3
sk. myosin + nem. actin ppt	17.4	23.0
sk. myosin + nem. actin sup	4.6	4.2
sk. myosin + nem. actin ppt + nem. actin sup	28.2	7.2
sk. myosin + sk. actin + nem. actin sup	21.2	12.5
sk. myosin + sk. actin	23.2	27.5

* Nematode actin fraction was centrifuged at 10^5xg for 3 hr and the precipitate was suspended by the original volume of the buffer (see "EXPERIMENTAL PROCEDURE").

the column chromatography on DEAE-cellulose (2). Figure 2 shows the ATPase activity of actomyosin reconstituted from skeletal myosin and nematode actin fraction. The ATPase activity of myosin itself was 2.7 min^{-1}. The ATPase activity was greatly activated to 21 s^{-1} in the presence of 1 mM $CaCl_2$ (●), but did not show activation by actin fraction in the absence of Ca (○).

Actin fraction was ultracentrifuged in 0.5 M KCl to separate F-actin and regulatory proteins. As shown in Fig.1, the precipitate after the centrifugation contained only F-actin (lane d) while supernatant contained tropomyosin with MW 40 K (22), and other minor proteins.

We investigated the Ca^{2+} regulation of myosin Mg^{2+}-ATPase activity by the precipitate (actin) and the supernatant (regulatory proteins). The precipitate caused the activation of the Mg^{2+}- ATPase activity of skeletal myosin in a Ca^{2+}-insensitive manner (17.4 and 23.0, respectively in the presence of Ca^{2+} or EGTA). On the other hand, the supernatant alone did not stimulate the activity (4.6 and 4.2 min^{-1}, respectively). When the supernatant was mixed with nematode actin (precipitate) or skeletal muscle actin, the Mg^{2+}ATPase activity of skeletal muscle myosin was activated in a Ca^{2+} dependent manner (28.2 and 7.2 min^{-1}) by nematode actin + supernatant and (21.5 and 12.5 min^{-1}) by skeletal muscle actin + supernatant.

Mg^{2+}-ATPase activities of nematode C.elegans myosin in the presence and absence of Ca^{2+} ions were measured in the combination of rabbit skeletal actin and chicken gizzard NTM. Table I shows that the Mg^{2+}-ATPase activity of nematode myosin was activated by rabbit skeletal actin about 20 times, but Ca^{2+}

TABLE II. Ca^{2+}-sensitivity of actin activated ATPase activity of nematode myosin. ATPase activities were assayed in the solution containing 0.05 mg/ml nematode myosin, 1.46 mg/ml rabbit skeletal pure F-actin, 0.8 mg/mk chicken gizzard "native" tropomyosin (NTM), 50 mM KCl, 2 mM $MgCl_2$, 0.5 mM ATP, 20 mM imidazole-HCl (pH 7.0), and 1 mM $CaCl_2$ or 2.5 mM EGTA, at 20 °C.

Condition	Activity (min^{-1})	
	+ Ca	+ EGTA
nematode myosin	0.4	0.4
nem. myosin + skeletal F-actin	7.7	7.6
nem. myosin + sk. F-actin + gizzard NTM	10.5	8.4

ions had no effect on Mg^{2+}-ATPase activities of both nematode myosin alone (0.4 min^{-1}) and that of skeletal actin-nematode myosin (7.7 min^{-1}).

Smooth muscle actomyosin ATPase reaction was controled via a Ca^{2+}-dependent phosphorylation and dephosphorylation of myosin light chain. Gizzard NTM prepared by Ebashi (18) has light chain kinase activity and can regulate the smooth muscle actomyosin ATPase reaction (23). The effect of NTM on nematode actomyosin ATPase activity was examined (Table II). Gizzard NTM caused slight enhancement of activity of skeletal acto-nematode myosin ATPase from 7.6 - 7.7 to 10.5 and 8.4 min^{-1}, respectively in the presence or absence of Ca^{2+} ions.

DISCUSSION

The presence of both actin- and myosin- linked calcium regulatory system were suggested in the nematode Ascalis lumbricoides (24,25) and Caenorhabditis elegans (22) on the basis of competitive assay using crude actomyosin fraction and pure skeletal myosin and actin. However the ATPase activity of actomyosin depends on the progress of the superprecipitation and does not simply depends on the concentration of actin and myosin.

We investigated the Ca^{2+}-dependent regulation in C.elegans muscle with purified myosin and actin fraction separated from crude actomyosin by DEAE-cellulose chromatography (2). The actin fraction activated the Mg^{2+}-ATPase activity of rabbit skeletal myosin in a Ca^{2+}-dependent manner (Fig.2). this regulatory actin fraction was separated into pure actin and regulatory proteins by

centrifugation in 0.5 M KCl (Fig.1). The regulatory proteins from C.elegans acted both on pure rabbit skeletal actin and on nematode actin in a similar manner as tropomyosin-troponin. A troponin-like protein has been isolated from body wall muscle of Ascaris lumbricoides, and separated into three component by Obinata et al.(26). We are now analyzing the troponin subunits included in the troponin fraction. However no common peptide have been observed in our preparation (data not shown). The Mg^{2+}-ATPase activity of myosin from C.elegans was remarkably increased about 20 times by rabbit skeletal actin in a Ca^{2+}-dependent manner (Table I). this result does not agree with that of Harris et al.(22). However the activation of myosin ATPase by pure actin was only slight in their system. The concentration of Ca^{2+} had no effect on the Mg^{2+}-ATPase activity of rabbit acto-nematode myosin, even when myosin was phosphorylated by gizzard NTM cotaining myosin light chain kinase. These results suggest that a myosin-linked Ca^{2+} regulatory system is lacking in C.elegans mascle.

The majority of the 30 known muscle genes of C.elegans were identified by analizyng uncoordinated mutants (unc mutations) or suppressors of unc mutations. However the products of these genes have not yet been revealed, exept unc-54 (myosin heavy chain) and unc-15 (paramyosin). We hope that the muscle regulatory protein genes of C.elegans will be identified and research for regulation of muscle gene expression will be developed.

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CONCLUSION

Unc-54 myosin of the nematode Caenorhabditis elegans was purified by an improved method. The primary structure of this myosin has been analyzed from the DNA sequence. The ATPase characteristics of nematode myosin was similar to that of skeletal muscle myosin though the amino acid sequence was quite different. The structure of this myosin studied from electron microscopy and protease digestion indicated that head-tail junction and center of the tail are flexible, though the location of flexible region are different from that estimated from the DNA sequence. This myosin forms long filament even in the absence of paramyosin. We found that Unc-54 myosin also has non-identical two heads as skeletal muscle myosin; one head forms M-ADP-P complex while other head forms M-ATP complex. The reactivity of specific lysine residue in heads B and A are also different as skeletal muscle myosin. Therefore, we concluded that the structure of two heads of unc-54 myosin are different from each other.

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