



HOKKAIDO UNIVERSITY

Title	TWO CALCIUM REGULATORY SYSTEMS IN SQUID MANTLE MUSCLE
Author(s)	KONNO, Kunihiko
Citation	MEMOIRS OF THE FACULTY OF FISHERIES HOKKAIDO UNIVERSITY, 29(1), 1-62
Issue Date	1982-11
Doc URL	https://hdl.handle.net/2115/21873
Type	departmental bulletin paper
File Information	29(1)_P1-62.pdf



TWO CALCIUM REGULATORY SYSTEMS IN SQUID MANTLE MUSCLE

By

Kunihiko KONNO

*Faculty of Fisheries, Hokkaido University,
Hakodate, Japan*

Contents

	page
I Introduction	3
II Methods and materials	4
1 Preparative methods for muscle proteins	4
1-A Myosin B and myosin from squid mantle muscle	4
1-B Actin from squid mantle muscle	5
1-C Native tropomyosin from squid mantle muscle	5
1-D Troponin and tropomyosin from squid mantle muscle	5
1-E Myosin, desensitized myosin and EDTA-LC from scallop adductor muscle	6
1-F Light chains from squid mantle muscle	6
1-G Myosin and actin from rabbit skeletal muscle	7
1-H Glycerinated muscle fibers from scallop adductor muscle	7
2 Assay method for biological and physicochemical properties	7
2-A Assay for ATPase activity	7
2-B Measurement of superprecipitation	8
2-C SDS gel, urea gel and disc gel electrophoreses and densitometry	8
2-D UV absorption spectra of light chains and their divalent cation induced difference spectra	8
2-E Calcium binding of myosin and light chains	9
2-F Quantitative determination of protein concentration	9
2-G Tension determination of glycerinated muscle fibers from scallop muscle ..	9
3 Materials and reagents	9
III Results	10
1 Actin-linked Ca regulation	11
1-A Native tropomyosin in squid muscle	11
1-A-a Ca sensitivity of squid acto-skeletal myosin	11
1-A-b Isolation of native tropomyosin from squid muscle	11
1-A-c A comparison between native tropomyosin from rabbit and squid muscle	12
1-A-d Binding of squid native tropomyosin to rabbit F-actin	14
1-B Troponin and tropomyosin in squid muscle	16
1-B-a Isolation of troponin and tropomyosin from squid muscle	16
1-B-b SDS gel electrophoretic patterns of squid troponin and tropomyosin ..	16
1-B-c Effect of squid troponin, tropomyosin and their complex on the super-precipitation of skeletal actomyosin	17

This work was submitted in partial fulfillment of the requirements for the Doctor's degree in Fisheries Science at Hokkaido University in 1981.

1-B-d	A comparison between troponins from rabbit and squid muscles	18
1-B-e	Binding of squid troponin and tropomyosin to F-actin	20
2	Myosin-linked Ca regulation	21
2-A	Ca sensitive myosin from squid mantle muscle	21
2-A-a	SDS gel electrophoretic patterns of squid myosin and myosin B	21
2-A-b	Ca sensitivity of skeletal acto-squid myosin	22
2-A-c	EDTA treatment of squid myosin	23
2-B	A comparison of Ca and Sr requirements for Mg-ATPase activity of myosin-linked and actin-linked regulations	23
2-B-a	Ca and Sr requirements for squid myosin B Mg-ATPase	23
2-B-b	Effects of squid and rabbit native tropomyosin on the Ca requirement of Mg-ATPase of squid myosin B	24
2-C	Regulatory light chains from squid myosin	25
2-C-a	Isolation of highly purified squid light chains	25
2-C-b	Effect of 2-mercaptoethanol on the patterns of squid light chains in disc gel electrophoresis	27
2-C-c	Effect of calcium on the patterns of squid light chains in disc gel electrophoresis	27
2-C-d	UV absorption spectra of isolated squid light chains and their changes induced by calcium or strontium ions	28
2-C-e	UV absorption spectrum of scallop EDTA-LC and the change induced by calcium or strontium ions	28
2-D	Desensitization and resensitization of scallop myosin	29
2-D-a	Desensitization of scallop myosin by EDTA treatment	29
2-D-b	Binding of squid light chains to scallop desensitized myosin	31
2-D-c	Resensitization of scallop desensitized myosin by squid light chain	32
2-D-d	Relationship between EDTA-LC (or squid LC-2) content of myosin and Ca sensitivity of acto-scallop myosin	33
2-D-e	Competitive binding of EDTA-LC and squid LC-2 to scallop desensitized myosin	34
2-E	Calcium binding of myosin and regulatory light chains	35
2-E-a	Calcium binding of isolated squid LC-2 and scallop EDTA-LC	35
2-E-b	Calcium binding of squid and scallop myosin	36
2-E-c	Effect of desensitization and resensitization on the calcium binding of scallop myosin	37
2-F	Calcium sensitivity of squid myosin alone	38
2-F-a	Ca requirement for activation of Mg-ATPase of squid myosin alone	38
2-F-b	Effects of temperature and pH on Ca sensitivity of squid myosin	39
2-F-c	Effect of KCl concentration on Ca sensitivity of squid myosin	40
2-F-d	ITP as substrate for squid myosin	41
2-F-e	Effect of urea on Ca sensitivity of squid myosin	42
2-F-f	Effect of tryptic digestion of squid myosin on Ca sensitivity of its ATPase	44
2-F-g	Ca sensitivity of scallop myosin alone and its loss by EDTA treatment	45
2-F-h	Effects of desensitization and resensitization on superprecipitation ability of acto-scallop myosin	49
2-G	ATP concentration dependence of ATPase and superprecipitation	49
2-G-a	ATP concentration dependence of superprecipitation and ATPase of myosin B of squid and scallop	49
2-G-b	ATP concentration dependence of Mg-ATPase activity of squid and scallop myosin	50
2-G-c	ATP concentration dependence of Mg-ATPase activity of desensitized and resensitized scallop myosin and actomyosin	50

2-H	Tension development of glycerinated muscle fibers of scallop adductor muscle	51
2-H-a	Effect of EDTA treatment of glycerinated fibers on the Ca sensitivity in tension development	51
2-H-b	Relationship between EDTA-LC content in fibers and Ca sensitivity of tension development in the presence of calcium	53
2-H-c	Effect of regulatory light chain on Ca sensitivity and on tension development in the presence of calcium	53
IV	Discussion	54
V	Summary	58
VI	Acknowledgements	60
VII	References	60

I. Introduction

It is well known that two different control systems regulate the activity of various muscles¹⁾²⁾. In vertebrate muscle, troponin and tropomyosin are apparently the only regulatory proteins and the control is therefore actin-linked³⁾. However, the mechanism of regulation in molluscan muscle differs from that in vertebrate muscle. In 1970, KENDRICK-JONES *et al.*²⁾ found myosin-linked Ca regulation in scallop muscle, and also found that one of the light chain components of scallop myosin acts as a regulatory subunit⁴⁾⁵⁾.

In both types of regulation, contraction is triggered by small amounts of calcium ions. The resting state is maintained because actin and myosin are unable to interact in the absence of calcium, and this occurs by blocking of sites either on actin or myosin. Despite this similarity in function, the interaction between actin and myosin is prevented differently in the two regulatory systems as the two systems contain different components. These two types of components (troponin in actin-linked and regulatory light chain in myosin-linked) cannot be related to each other in any simple fashion, and it is very unlikely that the one regulatory system could have evolved directly from the other.

A comparative study may give insights into the way the two regulatory mechanisms evolved as well as clarify certain functional differences between the various muscles. LEHMAN and SZENT-GYÖRGYI⁶⁾ extensively investigated the distribution of regulatory systems, and categorized vertebrate muscle as having an actin-linked regulation system, and molluscan muscle as having a myosin-linked regulation system.

In the present report, a study is described of the Ca regulation system in the mantle muscle of squid and in the adductor muscle of scallop. The author was able to isolate not only Ca sensitive squid myosin but also squid troponin as well as native tropomyosin in a pure form. It can be concluded, therefore, that squid mantle muscle has both a myosin-linked regulatory system and a actin-linked regulatory system, although the myosin-linked regulation appears to be predominant in preparations of squid myosin B.

A further study is conducted on the isolation and characterization of the regulatory subunit in squid myosin (regulatory light chain). In the process of functional characterization of squid regulatory light chain, the author found some important differences from those described by KENDRICK-JONES *et al.*⁵⁾ in the

functional role of the regulatory light chain.

II Methods and materials

1 Preparative methods for muscle proteins

1-A Myosin B and myosin from squid mantle muscle

Preparative methods for squid myosin B and for myosin are outlined in Chart I. The mantle muscle was quickly excised from a live squid, blended with 0.16 M KCl, 20 mM Tris-HCl (pH 7.5), 0.1 mM PMSF and 10 mM 2-mercaptoethanol and centrifuged to remove the supernatant. The muscle residue was washed several times with the same solution, then extracted with 3 vols of WEBER-EDSALL solution containing 1 mM ATP, 0.1 mM PMSF, and 10 mM 2-mercaptoethanol at 0°C for 24 h. The extract was centrifuged and diluted with 4 vols of 1 mM NaHCO₃. The precipitate thus formed was collected by centrifugation, redissolved in 0.2 M MgCl₂, 5 mM ATP, 1 mM EGTA, 10 mM 2-mercaptoethanol, and 10 mM phosphate buffer (pH 6.4), and centrifuged at 10,000 × g for 30 min to remove insoluble materials.

- Squid mantle muscle
- mince at 0–4°C
 - wash with 0.16 M KCl, 0.1 mM PMSF, pH 7.5
 - extract with WEBER-EDSALL solution containing 1 mM ATP, 0.1 mM PMSF and 0.1 mM DTT for 2 h at 0–4°C
 - centrifuge at 14,000 × g for 20 min
- Supernatant (myosin B)
- dilute KCl to 50 mM by adding 1 mM NaHCO₃
 - centrifuge at 10,000 × g for 10 min
- Precipitate
- suspend in 0.2 M MgCl₂, 1 mM EGTA, 10 mM sodium phosphate pH 6.4
 - add 10 mM ATP
 - centrifuge at 80,000 × g for 90 min
- Supernatant
- dilute MgCl₂ to 15 mM by adding 1 mM NaHCO₃
 - centrifuge at 10,000 × g for 10 min
- Precipitate
- dissolve into 0.25 M KCl, 0.1 mM DTT, pH 7.5
 - centrifuge at 80,000 × g for 60 min
- Supernatant
- dilute KCl to 50 mM by adding 1 mM NaHCO₃
 - centrifuge at 14,000 × g for 10 min
- Supernatant
- add 10 mM MgCl₂
 - keep at 0–4°C for 30 min
 - centrifuge at 14,000 × g for 10 min
- Precipitate
- dissolve into 0.5 M KCl, 0.1 mM DTT, pH 7.5
 - dialyze against 0.5 M KCl, 0.1 mM DTT, 20 mM Tris-maleate pH 7.5
 - centrifuge at 100,000 × g for 60 min
- Supernatant (Myosin)

Chart I Preparative method of myosin from squid muscle

ATP (5 mM) was added to the clear supernatant, and the mixture was centrifuged at $80,000 \times g$ for 90 min, thus removing the actin precipitate. The supernatant was diluted with 13 vols of 1 mM NaHCO_3 . The myosin precipitate was collected by centrifugation, redissolved in 0.25 M KCl, 10 mM Tris-maleate (pH 7.0), and 10 mM 2-mercaptoethanol, and centrifuged at $100,000 \times g$ for 60 min, removing insoluble materials. The supernatant was diluted with 4 vols of 1 mM NaHCO_3 , and 10 mM MgCl_2 was then added⁷⁾. The myosin precipitate was collected by centrifugation, dissolved in 0.5 M KCl, 20 mM Tris-maleate (pH 7.5), and 0.2 mM DTT, and stored at 0°C.

1-B Actin from squid mantle muscle

Actin was prepared from the mantle muscle of squid by the method of SPUDICH and WATT⁸⁾. Crude G-actin was extracted from the acetone-dried muscle powder with 20 vols of 0.5 mM ATP, 0.5 mM 2-mercaptoethanol, 0.2 mM CaCl_2 , and 2 mM Tris-HCl (pH 7.6) for 30 min at 0°C. G-actin in the extract was polymerized by adding 0.6 M KCl, 2 mM MgCl_2 , and 10 mM Tris-HCl (pH 7.6) and leaving the mixture stand for 1 h at room temperature (25°C). F-actin was collected by ultracentrifugation at $80,000 \times g$ for 180 min. The F-actin pellet was then dialyzed against the extraction solution, thus depolymerizing F-actin into G-actin. The G-actin solution was clarified by centrifugation at $100,000 \times g$ for 120 min, and the clear supernatant of G-actin was repolymerized into F-actin.

1-C Native tropomyosin from squid mantle muscle

Squid native tropomyosin was prepared from mantle muscle by the method of EBASHI³⁾. The squid mantle muscle was blended with 0.16 M KCl, 20 mM Tris-HCl (pH 7.5) containing 0.1 mM PMSF, 10 mM 2-mercaptoethanol, and centrifuged to obtain muscle residue. This washing procedure was repeated several times. The washed residue was extracted with 10 mM 2-mercaptoethanol and 0.1 mM PMSF for a day at 0°C. The viscous supernatant of native tropomyosin was obtained by centrifugation, and fractionated by ammonium sulfate slating-out. The fraction precipitating between 45–65% saturation was collected by centrifugation, dissolved in a small volume of 1 mM NaHCO_3 , and dialyzed against the same solution. The dialyzed solution of native tropomyosin was then clarified by centrifugation at $100,000 \times g$ for 60 min.

1-D Troponin and tropomyosin from squid mantle muscle

Squid native tropomyosin was separated into troponin and tropomyosin by the method of EBASHI⁹⁾. The squid native tropomyosin solution in 0.4 M LiCl was brought to pH 4.7 with 0.5 N HCl at 0°C, and then centrifuged. The supernatant containing troponin was adjusted back to pH 7.5. For further purification, the supernatant was chromatographed on a column of DEAE-cellulose. Troponin was eluted with KCl between 0.16 M and 0.18 M. Squid tropomyosin was prepared from the precipitate by placing native tropomyosin in 0.4 M LiCl at pH 4.7. It was purified by repeating the isoelectric precipitation at pH 4.7 in 0.4 M LiCl, and by precipitation with ammonium sulfate between 50 and 55% saturation.

1-E Myosin, desensitized myosin and EDTA-LC from scallop adductor muscle

Scallop myosin (myosin B or myofibrils) was prepared from scallop adductor striated muscle by the method used for squid myosin preparation¹⁰⁾. Scallop desensitized myosin was prepared by the method of SZENT-GYÖRGYI *et al.*⁴⁾ with a slight modification. Scallop myosin was kept suspended for 15 min in an ice-cooled solution of 10 mM EDTA, 30 mM KCl, and 10 mM Tris-HCl (pH 7.5). The suspension was then centrifuged at 6,000 × g for 10 min, and the supernatant and the sediment were separated. The EDTA-treatment procedure was repeated several times. The pellet of desensitized myosin was dissolved in 0.5 M KCl, 20 mM Tris-maleate (pH 7.5), and 1 mM DTT. The supernatants were combined and used to isolate scallop EDTA-LC by ammonium sulfate salting-out. The fraction precipitating between 50 and 100% saturation with ammonium sulfate was collected.

1-F Light chains from squid mantle muscle

Two types of light chain subunits of squid myosin were dissociated from large polypeptide chains (heavy chains) by the method of HOLT and LOWEY¹¹⁾. Preparative method of squid light chains is outlined in Chart II. Squid myosin (10–20 mg/ml) was kept at 25°C for 30 min in 4 M urea, 0.25 M KCl, 25 mM Tris-HCl

- Squid myosin
- 4 M urea, 0.25 M KCl, 2.5 mM EDTA, 2.5 mM DTT, and 25 mM Tris-HCl, pH 8.0 for 30 min at 25°C
 - dilution to 50 mM KCl
 - centrifugation
- Supernatant
- DEAE-cellulose
- 20 mM Tris-HCl, pH 8.0, 0.1 mM DTT
 - elution with 1 M KCl
 - dialysis against 0.15 M KCl, 20 mM Tris-HCl, pH 8.0, 0.1 mM DTT
 - centrifugation
- Supernatant
- ammonium sulfate salting-out
- Precipitate
- between 50 and 100% saturation
 - dialysis against 0.15 M KCl, 20 mM Tris-HCl, pH 8.0, 0.1 mM DTT
- Dialyzate
- Sephadex G-75 (2.5 × 90 cm)
- 0.15 M KCl, 20 mM Tris-HCl, pH 8.0, 0.1 mM DTT
- Light chains fraction
- DEAE-Sephadex A 25 (1 × 80 cm)
- 0.15–0.2 M KCl, 20 mM Tris-HCl, pH 8.0, 0.5 mM DTT
- LC-2 (→ 0.23 M KCl)
- LC-1 (→ 0.25 M KCl)

Chart II. Preparative method for squid light chain

(pH 8.0), and 20 mM 2-mercaptoethanol. The heavy chains precipitated on dilution with 10 vols of cold water, and the light chains were collected in the supernatant on centrifugation. In order to concentrate the light chains, the supernatant was applied to a DEAE-cellulose column, removing the unretained fraction, and the column was eluted with a small volumes of 1 M KCl containing 20 mM Tris-HCl (pH 8.0) and 10 mM 2-mercaptoethanol. The concentrated light chain solution thus obtained was dialyzed against 0.15 M KCl, 20 mM Tris-HCl (pH 8.0), and 10 mM 2-mercaptoethanol, and insoluble materials formed were removed by centrifugation at $20,000 \times g$ for 30 min. The supernatant containing light chains was then fractionated by ammonium sulfate salting-out. The fraction precipitating between 50 and 100% saturation with ammonium sulfate was collected. This fraction was fairly free from paramyosin, actin, tropomyosin, and troponin. If necessary, especially when it was obtained from myosin B or myofibrils, the light chain fraction was further purified by Sephadex G-75 gel filtration in 0.15 M KCl and 20 mM Tris-HCl (pH 8.0). In the gel filtration, paramyosin, actin, and troponin-tropomyosin complex were eluted in the void volume, and the light chains were retained, thus being separated. Light chains thus obtained in a pure form were chromatographed on DEAE-Sephadex A25 column¹²⁾ to obtain the two types of light chains separately. A long column of 1×80 cm, a shallow gradient of KCl concentration (from 0.15 to 0.3 M) and a slow rate of flow (10 ml/h) were used to facilitate the separation.

1-G Myosin and actin from rabbit skeletal muscle

Skeletal myosin was prepared from rabbit back and leg muscle by the method of CONNELL¹²⁾ with a slight modification. Rabbit actin was prepared by the method of SPUDICH and WATT⁸⁾.

1-H Glycerinated muscle fibers from scallop adductor muscle

Glycerinated muscle fiber bundles of scallop adductor muscle were prepared by the routine method of SZENT-GYÖRGYI *et al.*¹⁴⁾. Strips of scallop adductor muscles, approximately 2 mm \times 2 mm in section and 2-3 cm in length, were dissected out, tied with cotton threads to glass rods of the appropriate length, and immersed in a solution containing 50% glycerol, 5 mM phosphate buffer (pH 7.0), 4 mM MgCl₂, 2 mM EGTA and 1 mM 2-mercaptoethanol at 0°C. After overnight incubation at 0°C with one exchange of the solution, the preparation was immersed in the new solution and stored at -20°C for at least a month before use.

2 Assay method for biological and physicochemical properties

2-A Assay for ATPase activity

The actin-activated Mg-ATPase activity of myosin or that of myosin alone were assayed at 25°C by measuring Pi liberation. Phosphate was determined by the method of FISKE and SUBBAROW¹⁵⁾, except that *p*-methylaminophenol sulfate was used in place of 1,2,4-aminonaphtol sulphonic acid as a reducing reagent. In the ATP concentration dependence, an ATP regenerating system of pyruvate kinase and phosphoenol pyruvate (1 mM) was used to maintain the ATP concentration,

and ATPase activity was determined by measuring liberated pyruvate¹⁶). The standard reaction medium contained 20 mM Tris-maleate (pH 6.8), 30 mM KCl, 2 mM MgCl₂, 1 mM ATP, and 0.1 mM CaCl₂ or 0.5 mM EGTA. Ca sensitivity was defined as:

$$\text{Ca sensitivity} = [(A_{Ca} - A_E) / A_{Ca}] \times 100,$$

where A_{Ca} and A_E are Mg-ATPase activity in the presence of 0.1 mM CaCl₂ and 0.5 mM EGTA, respectively. The desired concentration of the free calcium ions was obtained by the addition of 0.2 mM CaCl₂ and appropriate concentrations of EGTA. Desired concentrations of free strontium ions were obtained by adding 0.5 mM EGTA and appropriate concentrations of SrCl₂. The stability constants adopted for estimation of the free ion concentrations were $5 \times 10^5 \text{M}^{-1}$ for the Ca-EGTA complex and $6 \times 10^2 \text{M}^{-1}$ for the Sr-EGTA complex¹⁷).

2-B Measurement of superprecipitation

Superprecipitation of actomyosin was induced at 20°C by adding 0.5 mM ATP to a reaction medium containing 30 mM KCl, 20 mM Tris-maleate (pH 6.8), 2 mM MgCl₂, and 0.1 mM CaCl₂ or 0.5 mM EGTA. The absorbance at 550 nm was measured with a Shimadzu UV 210A spectrophotometer.

2-C SDS gel, urea gel and disc gel electrophoreses and densitometry

SDS gel electrophoresis was carried out as described by WEBER and OSBORN¹⁸). Urea gel electrophoresis was conducted essentially as described by PERRIE *et al.*¹²). Gels contained 7.5% polyacrylamide, 8 M urea, and 0.375 M Tris-HCl (pH 8.9). The sample protein was kept at 37°C for 1 h in a solution of 8 M urea, 0.1 M Tris-HCl (pH 8.9), 5% 2-mercaptoethanol, and Bromophenol Blue as a marker. The running buffer contained 0.05 M Tris and 0.19 M glycine (pH 9.4). Disc gel electrophoresis was conducted as described by DAVIS¹⁹). Gels contained 7.5% polyacrylamide, 0.375 M Tris-HCl (pH 8.9), 10% 2-mercaptoethanol, and 1 mM CaCl₂ (+Ca) or 1 mM EGTA(-Ca). The running buffer was the same that used for urea gel electrophoresis. Gels were stained with 0.25% Coomassie brilliant blue or 0.5% Fast Green dissolved in 50% methanol and 9.2% acetic acid²⁰). Destaining was performed with 50% methanol and 9.2% acetic acid. The density of Fast Green stained protein bands was measured at 640 nm with a Shimadzu CS-900 dual wavelength TLC scanner.

2-D UV absorption spectra of light chains and their divalent cation induced difference spectra

The ultraviolet absorption spectra of myosin light chains and the calcium- or strontium-induced change in the UV absorption of light chains were determined at 20°C in a medium containing 0.1 M KCl, 20 mM histidine (pH 6.8), using a Shimadzu 210A spectrophotometer. In obtaining UV difference spectra, the reference cell contained 1 mM EGTA and the sample cell contained Ca-EGTA buffer or Sr-EGTA buffer²¹).

2-E Calcium binding of myosin and light chains

Calcium binding to myosin and to regulatory light chains were measured by an equilibrium dialysis method, using $^{45}\text{Ca}^{21}$. The equilibrium dialysis was conducted at 20°C in 0.3 M KCl, and 20 mM histidine buffer (pH 6.8). The free calcium concentration was set by using Ca-EGTA buffer¹⁷.

2-F Quantitative determination of protein concentration

Protein concentrations were determined by the biuret method, using bovine serum albumin fraction V as a standard²².

2-G Tension determination of glycerinated muscle fibers from scallop muscle

The isometric tension of muscle fiber bundles were determined as described by SIMMONS and SZENT-GYÖRGYI²³. The glycerinated muscle strip was shredded into a muscle bundle with forceps with thin and well fitted tips. The muscle bundle was immediately applied to the tenisometer. One end of the fiber bundle was fixed to an immobilized glass bar and other to transducer (UL-2, Shinkoh Tsushin) which was connected to a amplifier-recorder system. The attached fibers were rinsed in a solution containing 0.1 M KCl, 20 mM imidazole (pH 6.8), 5 mM MgCl_2 , 2 mM EGTA, and 0.3 mM DTT.

Two types of solutions were used in this study: a contracting solution (0.1 M KCl, 20 mM imidazole (pH 6.8), 5 mM MgCl_2 , 4 mM ATP, 0.2 mM CaCl_2 , 0.1 mM EGTA and 0.3 mM DTT) and a relaxing solution (0.1 M KCl, 20 mM imidazole (pH 6.8), 5 mM MgCl_2 , 4 mM ATP, 1 mM EGTA, and 0.3 mM DTT). Contraction was induced by changing the solution, in which the fibers were immersed, from a relaxing solution to a contracting solution. The temperature of the cell in which the fibers were was held at 20°C by circulating water under the cell.

For the EDTA-treatment of the fiber bundle attached to the tenisometer, fibers were soaked in a solution containing 0.1 M KCl, 20 mM Tris-HCl (pH 7.5), 10 mM EDTA, and 0.3 mM DTT at 20°C for various intervals. In order to resensitize the muscle fibers, thus treated with EDTA, they were soaked in a relaxing solution containing approximately 1.8 mg/ml of scallop EDTA-LC or squid LC-2 for various intervals.

3 Materials and reagents

The abbreviations used were as follow:

ATP(adenosine 5' triphosphate)

ITP(inosine 5' triphosphate)

EDTA(ethylenediamine tetraacetate)

EGTA(ethyleneglycol bis (β -aminoethyl)-N,N' tetraacetate)

DTT(dithiothreitol)

DTNB (5,5'-dithiobis(2-nitrobenzoate))

SDS(sodium dodecyl sulfate)

PMSF(phenylmethylsulfonyl fluoride)

DEAE-cellulose (diethylaminoethyl-cellulose)
 UV (ultraviolet)
 OD (optical density)
 LC (light chain component of myosin)
 HC (heavy chain component of myosin)
 NTM (native tropomyosin)
 TN (troponin)
 TM (tropomyosin)

The animals used were as follow:

rabbit (*Oryctolagus cuniculus domesticus*)
 squid (*Todarodes pacificus*)
 scallop (*Patinopecten yessoensis*)

The reagents purchased were as follow:

ATP (Daiichi pure chemicals Co. Ltd.)
 SDS (Nakarai chemical Ltd.)
 EDTA, EGTA (Dojin chemical Co. Ltd.)
 DEAE-cellulose (Brown Co.)
 Sephadex G-200, Sephadex G-75, DEAE-Sephadex A25 (Pharmacia fine chemicals)
 ITP, PMSF, PEP, trypsin, trypsin inhibitor (Sigma chemicals Co.)
 DTT, 2-mercaptoethanol, urea, ammonium sulfate, Fast Green, acrylamide (Wako pure chemical industry Ltd.)
⁴⁵Ca (Japan isotope center)

III Results

A competitive test devised by LEHMAN *et al.*⁶⁾²⁴⁾ was applied to the squid myosin B to determine whether the Ca regulatory system is myosin-linked or actin-linked. As shown in Table 1, the ATPase activity of the squid myosin B preparation ($\mu\text{mol Pi/min/mg}$ myosin B) was 0.559 in the presence of 0.1 mM CaCl_2

Table 1. Effect of skeletal F-actin and skeletal myosin on the Mg-ATPase of squid myosin B.

Mg-ATPase was assayed at 25°C in a medium containing 30 mM KCl, 20 mM Tris-maleate (pH 6.8), 2 mM MgCl_2 , 1 mM ATP and 0.1 mM CaCl_2 (+Ca) or 0.5 mM EGTA (-Ca), and 89 $\mu\text{g/ml}$ of squid myosin B (A), or 44 $\mu\text{g/ml}$ of squid myosin B and 35 $\mu\text{g/ml}$ of skeletal F-actin (B), or 44 $\mu\text{g/ml}$ of squid myosin B and 41 $\mu\text{g/ml}$ of skeletal myosin (C). In (B) and (C), F-actin and myosin were mixed with myosin B in 0.5 M KCl, 20 mM Tris-maleate (pH 7.5) before making up the assay medium.

	Mg-ATPase ($\mu\text{mol Pi/min/mg}$ myosin B)		
	+Ca	-Ca	Ca-sensitivity
(A) squid myosin B	0.559	0.003	99
(B) +F-actin	0.559	0.038	93
(C) +myosin	1.053	0.292	73

(+Ca), and 0.003 in the presence of 0.5 mM EGTA (-Ca). The two activities were unaffected by the addition of large amount of rabbit skeletal F-actin (34.6 μ g actin to 44.6 μ g of squid myosin B). The Ca sensitivity defined as described in "Methods and materials" was unaffected by the addition of a large amount of skeletal myosin (40.6 μ g myosin to 44.6 μ g of squid myosin B). It thus appeared that both myosin-linked and actin-linked Ca regulatory systems are present in squid myosin B. This view conflicts with that of LEHMAN and SZENT-GYÖRGYI⁶⁾, who categorized squid muscle in a group of muscles having only myosin-linked Ca regulation on the basis of the same competitive test. TSUCHIYA *et al.*²⁵⁾ also suggested the presence of actin-linked regulation in squid muscle on the basis of the same test.

1 Actin-linked Ca regulation

1-A Native tropomyosin in squid muscle

1-A-a *Ca sensitivity of squid acto-skeletal myosin*

The competitive test shown in Table 1 suggests that squid myosin B contains an actin-linked regulatory system as well as myosin-linked system. GOLDBERG and LEHMAN²⁶⁾ also recently reported the presence of actin-linked Ca regulatory complex. The author, therefore, investigated the actin-linked Ca regulation in squid muscle. Three different squid actin preparations were prepared for this purpose. They were: (A) pure F-actin obtained by the method of SPUDICH and WATT,⁸⁾ (C) crude F-actin *i.e.*, the whole extract from squid acetone-dried muscle powder polymerized by 0.1 M KCl, (pH 7.6) and 1 mM MgCl₂, and (B) partially purified F-actin, precipitated by ultracentrifugation of (C). Each of three preparations of squid actin was combined with rabbit skeletal myosin, which is Ca insensitive. Actomyosin thus reconstituted by using the F-actin (A) showed no Ca sensitivity in Mg-ATPase assay (Fig. 1A), whereas that reconstituted by using F-actin (C) showed a definite Ca sensitivity of 74% (Fig. 1C). That obtained using F-actin (B) also showed a Ca sensitivity of 50% (Fig. 1B).

SDS gel electrophoretic patterns of the three actin preparations are shown in Fig. 2. F-actin (A) was free from other proteins, crude F-actin (C) contained many other proteins, and F-actin (B) showed 5 protein bands including one actin band. These result reveal that four protein components are capable of associating with F-actin, thus precipitating with F-actin on ultracentrifugation. Accordingly, it is suggested that the four protein components may constitute squid native tropomyosin.

1-A-b *Isolation of native tropomyosin from squid muscle*

Native tropomyosin was isolated from the squid mantle muscle. The method for preparing rabbit skeletal native tropomyosin was successfully employed in the case of the squid muscle, though 10 mM 2-mercaptoethanol and 0.1 mM PMSF were present in the extracting medium, and the fraction precipitated between 45 and 65% saturation of ammonium sulfate. The SDS gel electrophoretic pattern of native tropomyosin thus isolated is shown later (Fig. 8). Four major bands with a few minor bands were detectable.

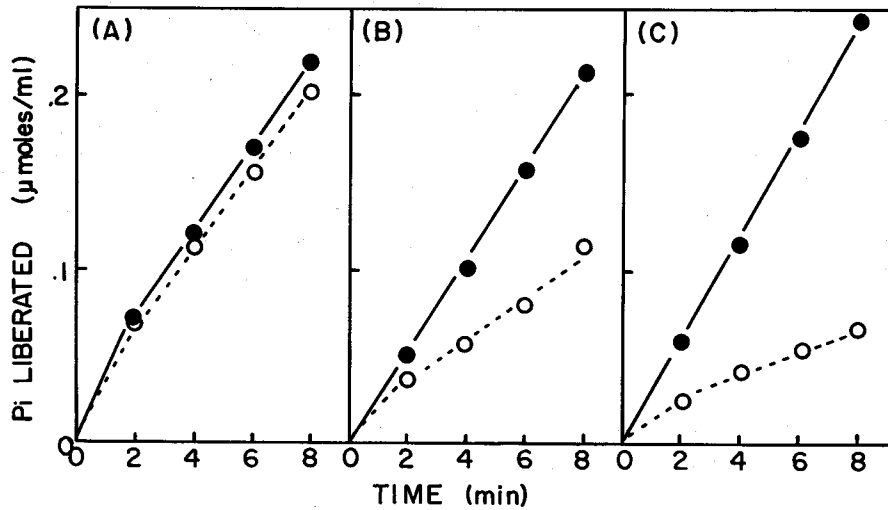


Fig. 1. Ca sensitivity of squid acto-skeletal myosin.

Actomyosins were reconstituted from rabbit skeletal myosin (82 $\mu\text{g/ml}$) and squid actin preparation of three different grades of purity; (A), actin (85 $\mu\text{g/ml}$) purified by the method of SPUDICH and WATT;⁹⁾ (C), crude actin (106 $\mu\text{g/ml}$) comprising whole extracts from acetone-dried squid muscle powder with 2 mM Tris-HCl (pH 7.6), 0.5 mM ATP, 0.5 mM 2-mercaptoethanol, and 0.2 mM CaCl_2 , polymerized with 0.1 M KCl 1 mM MgCl_2 ; (B), partially purified actin (98 $\mu\text{g/ml}$) precipitated by ultracentrifugation of (C). Mg-ATPase was assayed in the medium described in Table 1.

Squid native tropomyosin thus isolated was tested for its effect on the superprecipitation of rabbit skeletal actomyosin. As shown in Fig. 3A, skeletal actomyosin alone showed no Ca sensitivity. The time required to reach half-maximal turbidity (superprecipitation) ($\tau_{1/2}$) was 3.5 min in 0.1 mM CaCl_2 (●) as well as in 0.5 mM EGTA (○). On the other hand, in the presence of squid native tropomyosin (55 μg), $\tau_{1/2}$ for the superprecipitation of skeletal actomyosin (128 μg myosin plus 57 μg actin) was 5.5 min in 0.1 mM CaCl_2 (▲) and 9 min in 0.5 mM EGTA (Δ), thus showing Ca sensitivity. Purified squid actin was capable of replacing skeletal actin in the above test, that is, hybrid actomyosin (squid actin/skeletal myosin) alone showed no Ca sensitivity, $\tau_{1/2}$ being 4.5 min (●, ○). However, in the presence of squid native tropomyosin (55 μg), the hybrid (128 μg myosin plus 46 μg actin) showed Ca sensitivity, $\tau_{1/2}$ being 8 min in 0.1 mM CaCl_2 (▲) and 24 min in 0.5 mM EGTA (Δ). We, therefore, conclude that our preparation of squid native tropomyosin has the same Ca sensitizing action as skeletal native tropomyosin.

1-A-c A comparison between native tropomyosin from rabbit and squid muscle

The biological activities of squid native tropomyosin were compared with those of rabbit by measuring their inhibitory effect on skeletal actomyosin Mg-ATPase activity. As shown in Fig. 4, skeletal actomyosin alone was not

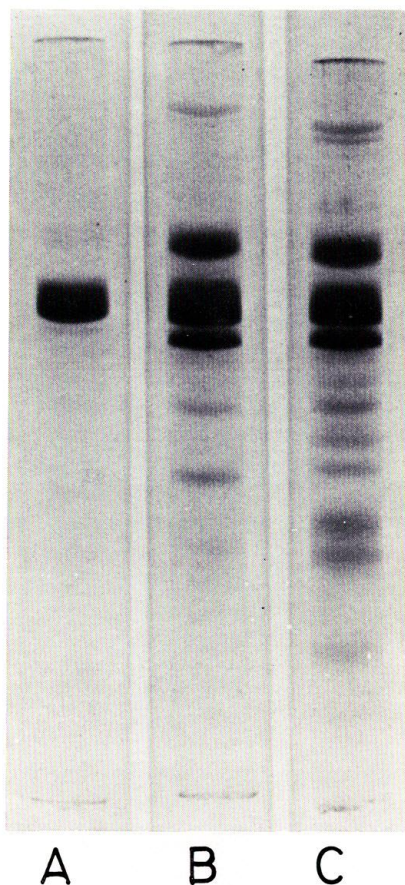


Fig. 2. SDS gel electrophoretic patterns of squid actin preparations.

A, B, and C were the actin preparations used in the experiments shown in Fig. 1A, B, and C, respectively. Approximately 10 μ g (A) or 20 μ g (B and C) of protein was loaded on each gel.

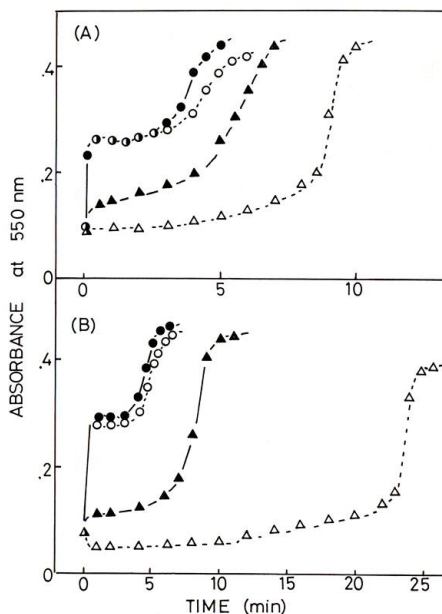


Fig. 3. Effect of squid native tropomyosin on the superprecipitation of reconstituted actomyosin.

Actomyosin was reconstituted from skeletal myosin (128 μ g/ml) and skeletal actin (57 μ g/ml) (A), or skeletal myosin (128 μ g/ml) and squid actin (46 μ g/ml). The circles refer to actomyosin alone, the triangles refer to actomyosin plus native squid tropomyosin (55 μ g/ml). The reaction was carried out at 20°C in a medium containing 30 mM KCl, 20 mM Tris-maleate (pH 6.8), 2 mM MgCl₂, 0.5 mM ATP and 0.1 mM CaCl₂ (●, ▲) or 0.5 mM EGTA (○, △).

sensitive to calcium. Addition of rabbit native tropomyosin conferred Ca sensitivity to this actomyosin as previously reported²⁷⁾, and squid native tropomyosin also conferred Ca sensitivity to actomyosin. Furthermore, squid native tropomyosin was distinguishable from that of rabbit in the following respect, namely that Mg-ATPase activity of actomyosin was inhibited by squid native tropomyosin not only in the absence of calcium but also in the presence of calcium. This was a characteristic of squid native tropomyosin which was not found in rabbit native tropomyosin (Fig. 4).

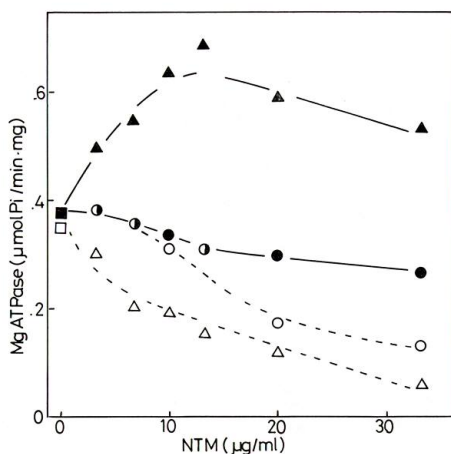


Fig. 4. Effects of native tropomyosins from squid and rabbit on the Mg-ATPase activity of actomyosin.

The ATPase activity of skeletal actomyosin was assayed in a medium containing 30 mM KCl, 20 mM Tris-maleate (pH 6.8), 2 mM MgCl₂, 1 mM ATP and either 0.1 mM CaCl₂ (closed) or 0.5 mM EGTA (open). Actomyosin alone (■, □); squid native tropomyosin (●, ○) and rabbit native tropomyosin (▲, △).

1-A-d Binding of squid native tropomyosin to rabbit F-actin

The binding ability of squid native tropomyosin to rabbit F-actin was measured by a centrifugation method. Squid native tropomyosin was mixed with F-actin in a medium of 0.1 M KCl, 20 mM Tris-maleate (pH 7.5) and maintained at 0°C for an hour. The mixture was then centrifuged at 100,000 ×g for three hours, and the F-actin pellet thus obtained was dissolved in SDS solution and applied to SDS gel electrophoresis. The results obtained were shown in Fig. 5. Beside the actin band, the four major protein bands were found to be identical to those of native tropomyosin. This result indicates that squid native tropomyosin is capable of binding to F-actin. The

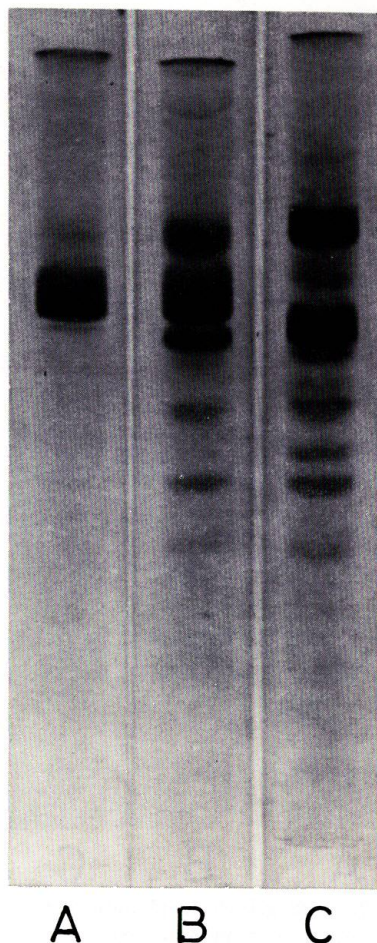


Fig. 5B. Binding of squid native tropomyosin to F-actin.

Squid native tropomyosin was mixed with rabbit F-actin in a weight ratio of 0.3:1 for native tropomyosin:F-actin in a medium of 0.1 M KCl, 20 mM Tris-maleate (pH 7.5), and maintained for an hour in ice-cold water. The mixture was then ultracentrifuged at 100,000 ×g for 3 hr, the pellet thus obtained was applied to a SDS gel rod. Squid native tropomyosin (C); pellet obtained after ultracentrifugation (B), and rabbit F-actin alone (A).

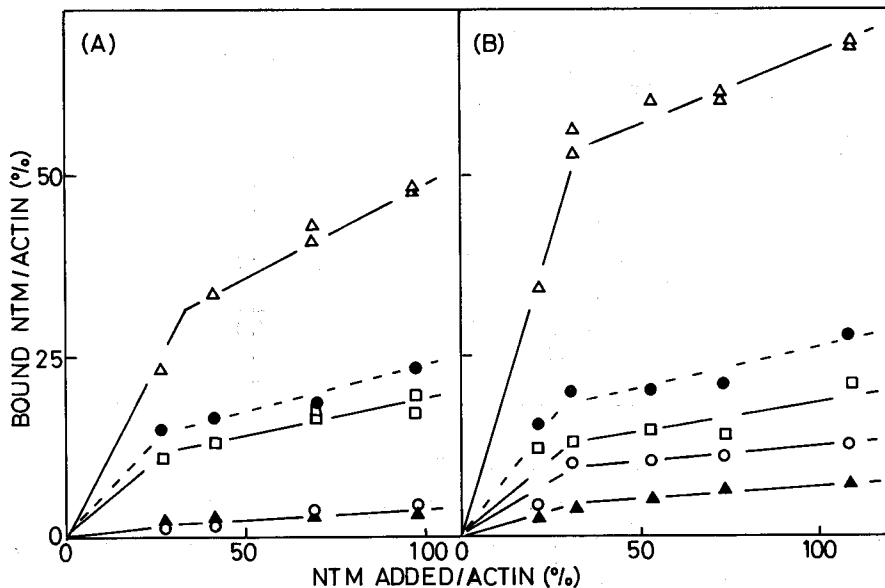


Fig. 6. Stoichiometric binding of native tropomyosin to F-actin.

Binding of native tropomyosin to F-actin was measured by the same method as in Fig. 5, except that various amounts of native tropomyosin were mixed. F-actin pellet combining native tropomyosin was separated and applied to a SDS gel and stained with Fast Green. The intensity of each subunit was determined. (A) squid native tropomyosin: (Δ), total; (\bullet), tropomyosin; (\square), 52,000 dalton component; (\circ), 28,000 dalton component, and (\blacktriangle), 24,000 dalton component, (B) rabbit native tropomyosin: (Δ), total; (\bullet), tropomyosin; (\square), troponin-T; (\circ), troponin-I, and (\blacktriangle), troponin-C.

stoichiometric binding of native tropomyosin to F-actin was then studied. As shown in Fig. 6, the maximum binding of native tropomyosin from squid or rabbit to F-actin was reached at around 30% addition (w/w) of native tropomyosin to F-actin. The amounts of subunits of native tropomyosin bound to F-actin were estimated from the density of the staining in the SDS gel electrophoretic patterns. Table 2 shows that the amounts of the 28,000 dalton and the 24,000 dalton components in squid native tropomyosin was found to be very small in the pellet precipitating with F-actin. Tropomyosin and the 52,000 dalton component were found in the pellet in an amount of about 1 mol per 7 mol of actin monomer. In addition, it was found that intact myofibrils from squid mantle muscle contained small amounts of the 28,000 dalton and the 24,000 dalton components. This fact suggests that the selective loss of these components did not occur during the preparation of squid native tropomyosin. These results, therefore, strongly indicates that squid native tropomyosin is composed of different (or irregular) subunit composition from that of rabbit native tropomyosin.

Table 2. Amounts of subunits in squid native tropomyosin bound to skeletal F-actin.

The amounts of subunits bound to F-actin were estimated from the data in Fig. 6.

	subunit	moles/7 moles of Actin
Rabbit	TM	1.0
	TN-T	1.4
	TN-I	1.4
	TN-C	1.0
Squid	TM	1.0
	TN-52,000	1.0
	TN-28,000	0.3
	TN-24,000	0.3

1-B Troponin and tropomyosin in squid muscle

1-B-a Isolation of troponin and tropomyosin from squid muscle

EBASHI's method⁹⁾ for the preparation of skeletal troponin was used with a slight modification to isolate squid troponin from squid native tropomyosin. Squid native tropomyosin was dissolved in 0.4 M LiCl, and the pH of the solution was adjusted to 4.7 by adding 0.5 N HCl. The supernatant (crude preparation of squid troponin) was collected by centrifugation. The crude preparation was further purified by DEAE-cellulose chromatography. The elution pattern is shown in Fig. 7. A major peak was eluted with KCl at a concentration around 0.17 M. This major peak was identified as squid troponin, on the basis of its SDS gel electrophoretic pattern (Fig. 8) and its Ca sensitizing activity. Squid tropomyosin was also isolated from native tropomyosin.

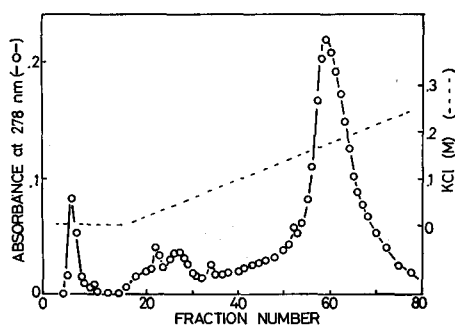


Fig. 7. DEAE-cellulose chromatography of squid troponin.

A solution of native tropomyosin in 0.4 M LiCl was brought to pH 4.7. Tropomyosin precipitated and was removed by centrifugation. The supernatant was readjusted at pH 7.5, dialyzed against 10 mM Tris-HCl (pH 7.5), and then applied (25.5 mg) to a column (0.8 × 15 cm) of DEAE-cellulose pre-equilibrated with the same buffer. The column was eluted with a linear gradient of KCl concentration. The flow rate was approximately 6 ml/h, and fractions of 3.8 ml were collected.

1-B-b SDS gel electrophoretic patterns of squid troponin and tropomyosin

SDS gel electrophoretic patterns of purified troponin, tropomyosin, and original native tropomyosin obtained from squid muscle are shown in Fig. 8. Purified squid troponin was identical with skeletal troponin showing three protein bands,

however squid troponin had a different mobility than rabbit skeletal troponin²⁸⁾²⁹⁾³⁰⁾ and also carp dorsal troponin²⁷⁾. It is rather similar to lobster troponin³¹⁾. The three bands of squid troponin corresponded to 52,000, 28,000, and 24,000 daltons.

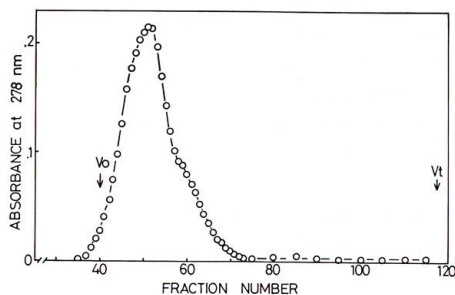
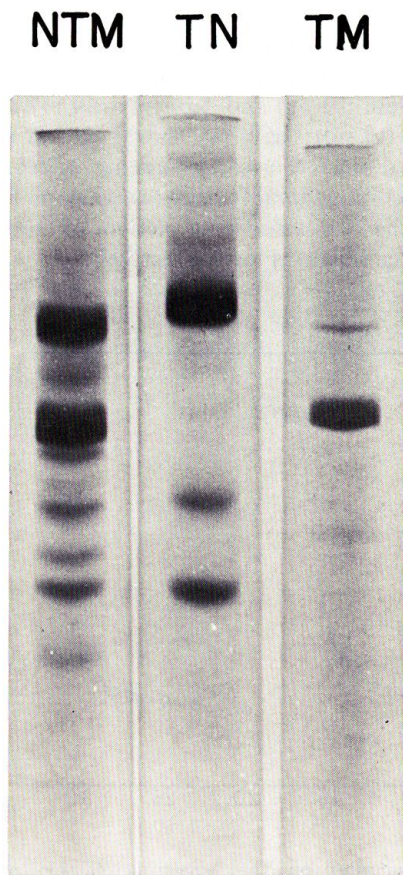


Fig. 9. Elution profile of squid troponin from Sephadex G-200 gel column.

Squid troponin in 0.1 M KCl, 20 mM Tris-HCl (pH 7.5) was applied to a Sephadex G-200 gel column pre-equilibrated with the same buffer. About 20 mg of troponin was applied and 4.8 ml/tube was collected. Void volume and bed volume were determined by using Blue dextran and ATP, respectively.

Fig. 8. SDS gel electrophoretic patterns of native tropomyosin, troponin and tropomyosin prepared from squid muscle.

NTM, native tropomyosin (20 μ g); TN, troponin (15 μ g); TM tropomyosin (5 μ g).

It seemed advisable to study whether the three subunits detected in SDS gel of squid troponin form a complex or not. Squid troponin was dissolved in 0.1 M KCl, 20 mM Tris-HCl (pH 7.5) and applied to a Sephadex G-200 gel column pre-equilibrated with the same buffer solution. The elution profile was shown in Fig. 9. Most of the protein applied was eluted after the void volume as a single fraction with an estimated molecular weight of 1×10^5 daltons. These results suggest that the three subunits in squid troponin form a complex under the above conditions.

1-B-c *Effect of squid troponin, tropomyosin and their complex on the superprecipitation of skeletal actomyosin*

The Ca sensitizing activity of squid troponin was examined by its effect on the superprecipitation of actomyosin in the presence of tropomyosin. Actomyosin was

reconstituted from rabbit skeletal myosin (157 μg) and squid purified actin (38 μg). As shown in Fig. 10A, no Ca sensitivity was detectable with actomyosin alone: $\tau_{1/2}$ was 4 min in the presence of 0.1 mM CaCl_2 (\bullet) as well as in its absence (\circ). Addition of squid troponin (51 μg) alone did not confer Ca sensitivity ($\blacktriangle, \triangle$). Addition of squid tropomyosin (47 μg) alone also failed to confer Ca sensitivity though it slightly reduced the rate of superprecipitation: $\tau_{1/2}$ was 9 min in the presence of calcium (\blacksquare) and 11.5 min in its absence (\square). On the other hand, Fig. 10B shows that the addition of both troponin (51 μg) and tropomyosin (47 μg) would confer Ca sensitivity: $\tau_{1/2}$ was 16 min in 0.1 mM CaCl_2 (\bullet) and 31 min in 0.5 mM EGTA (\circ). It can, therefore, be concluded that the Ca sensitizing effect of squid troponin-tropomyosin complex was similar in general features to that of the skeletal troponin-tropomyosin system, but the Ca sensitizing action of squid native tropomyosin or troponin-tropomyosin complex is less effective.

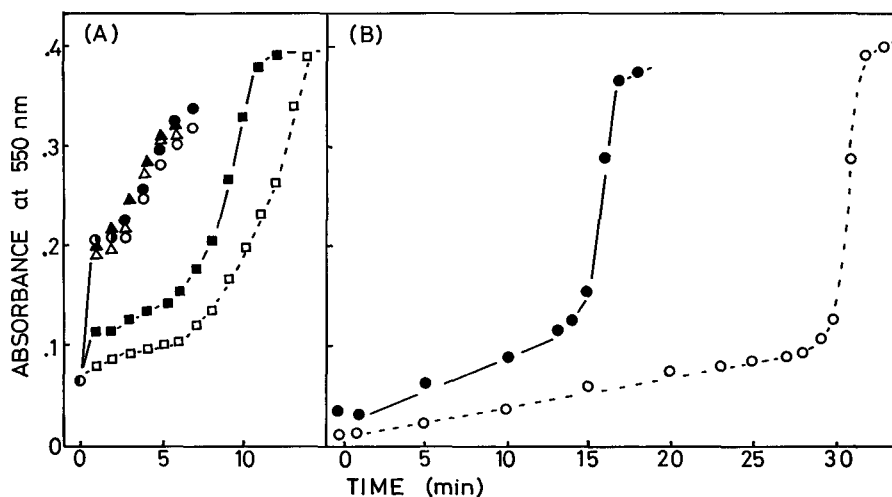


Fig. 10. Effect of squid troponin-tropomyosin complex on the superprecipitation of reconstituted actomyosin.

(A); Actomyosin reconstituted from rabbit skeletal myosin (157 $\mu\text{g}/\text{ml}$) and squid purified actin (38 $\mu\text{g}/\text{ml}$) with no addition (\bullet, \circ), or with squid troponin (51 $\mu\text{g}/\text{ml}$) ($\blacktriangle, \triangle$), or with squid tropomyosin (47 $\mu\text{g}/\text{ml}$) (\bullet, \circ). (B); Squid troponin (51 $\mu\text{g}/\text{ml}$)-tropomyosin (47 $\mu\text{g}/\text{ml}$) complex was added to the reconstituted actomyosin (\bullet, \circ). The reaction medium was as described in Fig. 3. Closed and open symbols denote 0.1 mM CaCl_2 ($\bullet, \blacktriangle, \blacksquare$) and 0.5 mM EGTA ($\circ, \triangle, \square$) media, respectively.

1-B-d A comparison between troponins from rabbit and squid muscles

The Ca sensitizing effects of hybrid and homologue troponin-tropomyosin complex formed between troponin and tropomyosin from squid and rabbit were then compared. The effect was estimated by measuring the inhibition of actomyosin Mg-ATPase activity by these complexes. Figure 11 showed the effect of complexes using squid troponin and squid tropomyosin or rabbit tropomyosin.

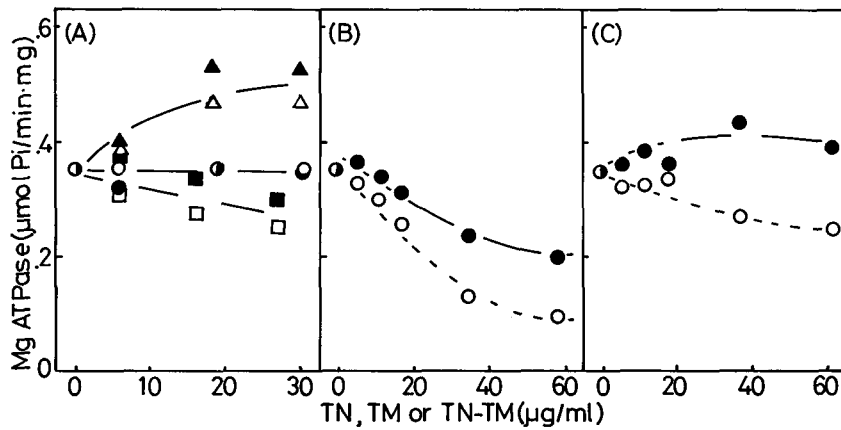


Fig. 11. Effects of hybrid and homologue complexes of troponins and tropomyosins from squid and rabbit muscle on the Mg-ATPase of actomyosin.

The ATPase was assayed in the same medium as in Fig. 4. (A): (●, ○), squid troponin alone; (▲, △), squid tropomyosin alone; and (■, □), rabbit tropomyosin alone. (B): squid troponin plus squid tropomyosin (1/1 by weight ratio). (C): squid troponin plus rabbit tropomyosin (1/1 by weight ratio).

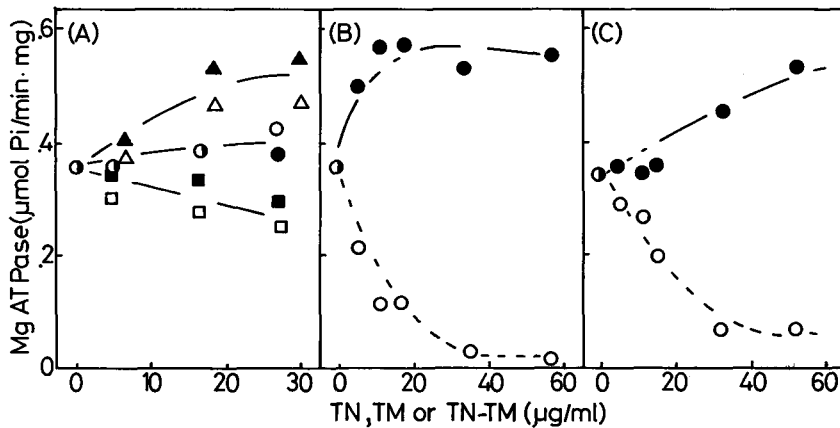


Fig. 12. Effects of hybrid and homologue complexes of troponins and tropomyosins from squid and rabbit muscles on the Mg-ATPase of actomyosin.

The ATPase was assayed in the medium as in Fig. 11. (A): (●, ○), rabbit troponin alone; (▲, △), squid tropomyosin alone; and (■, □) rabbit tropomyosin alone. (B): rabbit tropomyosin plus rabbit troponin (1/1 by weight ratio). (C): squid tropomyosin plus rabbit troponin (1/1 by weight ratio).

Neither of them also conferred Ca sensitivity on actomyosin (Fig. 11A). The squid troponin-tropomyosin complex conferred Ca sensitivity but to a very small degree. It should be noted that the Mg-ATPase activity was reduced even when

calcium was present in the system with the troponin-tropomyosin complex (Fig. 11B). These effects of the complex were similar to those obtained with native tropomyosin from squid mantle muscle. Replacement of squid tropomyosin by rabbit tropomyosin did not affect these general trends (Fig. 11C); the Ca sensitivity was slightly reduced with decreasing ATPase activity in the presence of calcium.

In contrast, as shown in Fig. 12, the troponin-tropomyosin complex which was formed using rabbit troponin did confer Ca sensitivity on actomyosin. This result indicated that squid tropomyosin was comparable to rabbit tropomyosin in this regard. Therefore, one can conclude that the relatively lower Ca sensitivity conferred by squid native tropomyosin was due to properties of squid troponin.

1-B-e *Binding of squid troponin and tropomyosin to F-actin*

The binding ability of squid troponin, tropomyosin, and their complex to skeletal F-actin was studied. Proteins were mixed in the medium of 0.1 M KCl, 20 mM Tris-HCl (pH 7.5), maintained at 0°C for an hour, centrifuged at $100,000 \times g$ for 3 h, and then the subunit composition of the pellet were analyzed by SDS gel electrophoresis. Figure 13 showed that squid tropomyosin as well as rabbit tropomyosin were bound to skeletal F-actin. Squid troponin alone did not bind to F-actin, while it bound to F-actin only when tropomyosin either from squid or rabbit was present in the system. It is, therefore, clear that the squid troponin preparation did not contain any subunit which was able to bind to F-actin, and that squid troponin initially bound to tropomyosin, forming a complex which was subsequently bound to F-actin. These binding features of troponin and tropomyosin were essentially identical with those of the rabbit troponin and tropomyosin system²⁸).

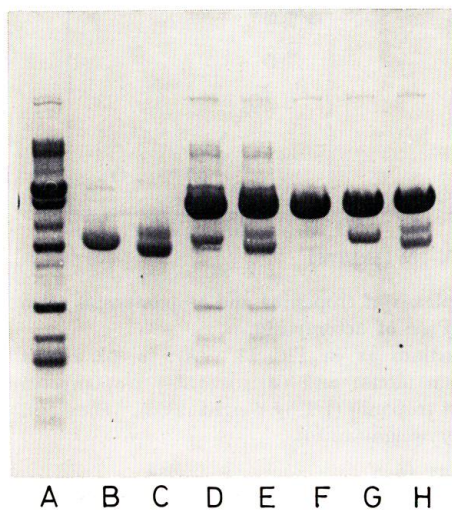


Fig. 13. Binding of squid troponin and tropomyosin to rabbit F-actin.

Binding of squid troponin and tropomyosin to rabbit F-actin was measured by the same method as described in Fig. 5. (A) Squid troponin preparation; (B) squid tropomyosin; (C), rabbit tropomyosin; (D) F-actin pellet with squid troponin and squid tropomyosin; (E), F-actin pellet with squid troponin and rabbit tropomyosin; (F), F-actin pellet with squid troponin; (G), F-actin pellet with squid tropomyosin; and (H), F-actin pellet with rabbit tropomyosin.

2 Myosin-linked Ca regulation

2-A Ca sensitive myosin from squid mantle muscle

The presence of myosin-linked Ca regulation in molluscan muscle, especially in scallop muscle has been already suggested by KENDRICK-JONES *et al.*²⁾. In the present study, the presence of myosin-linked Ca regulation system in squid myosin B was also suggested by a competitive test of squid myosin B with skeletal F-actin.

2-A-a SDS gel electrophoretic patterns of squid myosin and myosin B

SDS gel electrophoretic patterns of squid myosin B, myosin, and actin used in the present study are shown in Fig. 14. This preparation of myosin was free from actin contamination, though it contained a small amount of paramyosin. Two

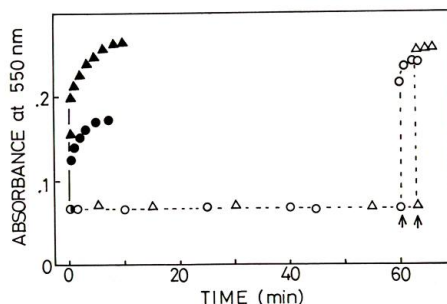
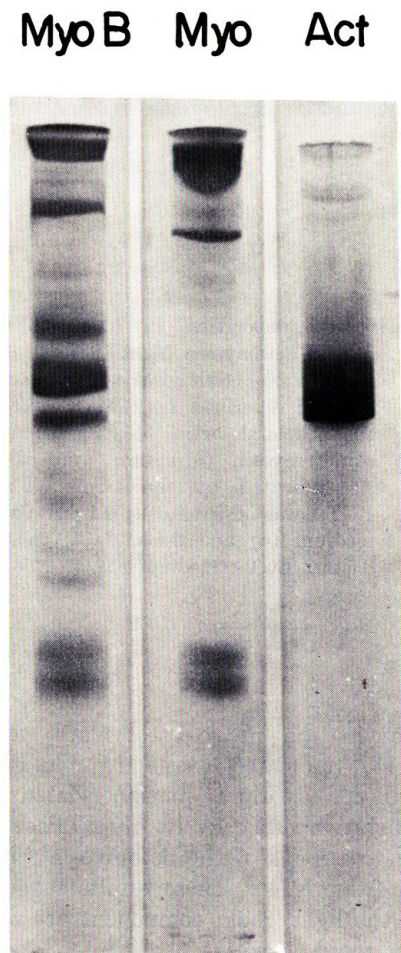


Fig. 15. Superprecipitation of hybrid and squid actomyosins.

Superprecipitation of reconstituted actomyosin was followed by measuring the absorbance at 550 nm. Actomyosin was reconstituted from squid myosin (202 $\mu\text{g}/\text{ml}$) and skeletal actin (107 $\mu\text{g}/\text{ml}$) (\bullet , \circ) or from squid myosin (202 $\mu\text{g}/\text{ml}$) and squid actin (175 $\mu\text{g}/\text{ml}$) (\blacktriangle , \triangle). The reaction was carried out at 20°C in a medium containing 30 mM KCl, 20 mM Tris-maleate (pH 6.8), 2 mM MgCl_2 , 0.5 mM ATP, and 0.1 mM CaCl_2 (\bullet , \blacktriangle) or 0.5 mM EGTA (\circ , \square). 0.5 mM CaCl_2 was added to the EGTA medium as indicated by the arrows.

Fig. 14. SDS gel electrophoretic patterns of myosin B, myosin, and actin prepared from squid mantle muscle.

10% polyacrylamide gels containing 0.1% SDS were prepared. Electrophoresis was carried out according to the method of WEBER and OSBORN.¹⁸⁾ Approximately 60 μg of myosin B (Myo B), 50 μg of myosin (Myo), and 30 μg of actin (Act).

light chain components with molecular weights of 15,000 and 17,000 were found in this preparation of squid myosin³²⁾³³⁾. Squid actin was virtually free from any other protein bands, and showed the same mobility as skeletal actin in SDS gel electrophoresis (data not shown)³⁴⁾.

2-A-b *Ca sensitivity of skeletal acto-squid myosin*

As shown in Fig. 15, actomyosin reconstituted from squid myosin (202 $\mu\text{g/ml}$) and skeletal purified F-actin (175 $\mu\text{g/ml}$) exhibited Ca sensitivity. Superprecipitation of skeletal acto-squid myosin in the presence of 0.1 mM CaCl_2 (●) occurred immediately after the addition of 0.5 mM ATP, but its occurrence in the presence of 0.5 mM EGTA (○) was greatly delayed (over 60 min). The addition of CaCl_2 to the EGTA medium (indicated by an arrow in Fig. 15) induced immediate superprecipitation. Essentially the same result was obtained when skeletal actin was replaced by purified squid F-actin (\blacktriangle). This squid F-actin was Ca insensitive, as shown in Fig. 1A.

Ca sensitivity of actomyosin reconstituted from squid myosin (87.0 $\mu\text{g/ml}$) and skeletal F-actin (45.8 $\mu\text{g/ml}$) was also demonstrated by Mg-ATPase assay. Figure 16 shows that Pi liberation proceeds rapidly in the presence of 0.1 mM CaCl_2 (●) but very slowly in its absence (○) and that, unlike actomyosin reconstituted

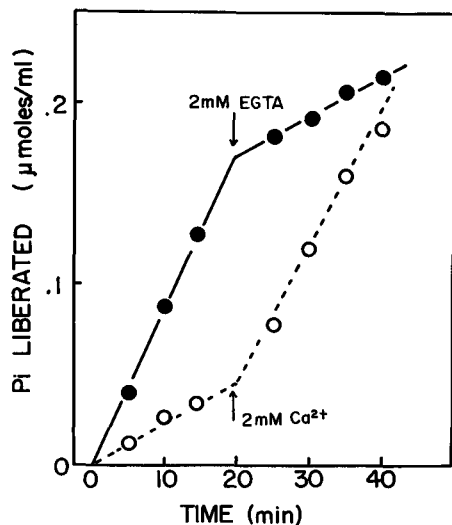


Fig. 16. Ca dependent Pi liberation by hybrid actomyosin Mg-ATPase.

ATPase activity was assayed in the medium described in Table I, except that skeletal actin (45.8 $\mu\text{g/ml}$) and squid myosin (87 $\mu\text{g/ml}$) were used. As indicated by the arrows, 2 mM EGTA was added to the 0.1 mM CaCl_2 medium (●) and 2 mM CaCl_2 to the 0.5 mM EGTA medium (○).

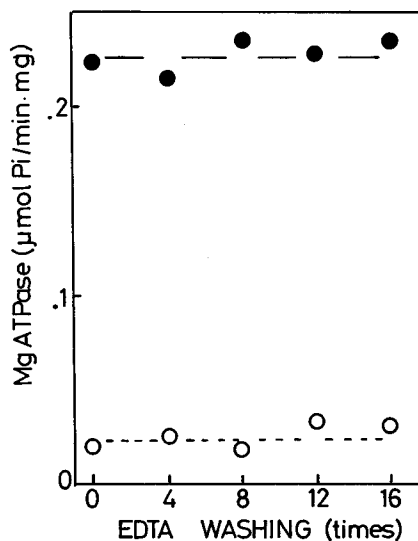
from gizzard myosin and actin³⁵⁾, Pi liberation in the presence of CaCl_2 was decreased by a subsequent addition of 2 mM EGTA (indicated by the arrow in Fig. 16). It is already known that chicken gizzard actomyosin is phosphorylated in the presence of calcium, so that the rate of liberation of Pi by its Mg-ATPase is unaffected by the addition of EGTA, whereas treatment of the actomyosin with phosphatase reduces the rate of Pi liberation from ATP. These results suggest that phosphorylation and dephosphorylation of the light chain component are not involved in the myosin-linked Ca regulation in squid muscle.

2-A-c EDTA-treatment of squid myosin

SZENT-GYÖRGYI *et al.*⁴⁾ reported that treatment of scallop myosin with EDTA in a low-salt medium caused the release of one of the light chain components, the so-called EDTA-LC, and resulted in the loss of its Ca sensitivity. However, the author found that EDTA-treatment had no effect on the Ca sensitivity of squid myosin. Squid myosin was treated 16 times, each time with 10 vols of 5 mM EDTA, 30 mM KCl, and 10 mM Tris-HCl (pH 7.5). Actomyosin was reconstituted from the EDTA-treated myosin (108 $\mu\text{g/ml}$) and skeletal actin (60 $\mu\text{g/ml}$), which was free from troponin and tropomyosin. The hybrid actomyosin thus reconstituted was found to be highly Ca sensitive (Fig. 17), that is, its Mg-ATPase activity ($\mu\text{mol Pi/min/mg}$ myosin) was 0.222 in the presence of 0.1 mM CaCl_2 (\bullet), whereas it was 0.02 in the presence of 0.5 mM EGTA (\circ). These activity values were essentially the same as those obtained with actomyosin reconstituted from untreated squid myosin and actin. No change in the SDS gel electrophoretic pattern of squid myosin was detectable upon EDTA-treatment (data not shown). The results suggesting that squid myosin was not able to be desensitized by treatment with EDTA agree with that reported by SZENT-GYÖRGYI *et al.*⁴⁾, using *Loligo siphone* muscle myosin.

Fig. 17. Effect of EDTA treatment on the Ca sensitivity of squid myosin.

Squid myosin was washed 16 times with 10 vols of 5 mM EDTA, 30 mM KCl, and 10 mM Tris-HCl (pH 7.5). Actomyosin was then reconstituted from EDTA-treated myosin (108 $\mu\text{g/ml}$) and skeletal actin (60 $\mu\text{g/ml}$). Mg-ATPase activity was assayed in the presence of 0.1 mM CaCl_2 (\bullet) or 0.5 mM EGTA (\circ) in the reaction medium described in Table I.



2-B A comparison of Ca and Sr requirements for Mg-ATPase activity of myosin-linked and actin-linked regulations

2-B-a Ca and Sr requirements in squid myosin B Mg-ATPase

The question arose as to which of the two regulatory systems is predominant in squid muscle. KENDRICK-JONES *et al.*²⁾ reported that scallop myosin B Mg-ATPase (myosin-linked) was fully activated only at higher calcium concentrations than rabbit skeletal myosin B Mg-ATPase. Initially, the Ca and Sr requirements

for Mg-ATPase of squid myosin B were studied by comparing them with those of rabbit myosin B and two hybrid actomyosin: rabbit actin/squid myosin (myosin-linked regulatory system) and crude squid actin/rabbit myosin (actin-linked regulatory system). As shown in Fig. 18, the Ca and Sr requirements of squid myosin B (●) were identical with those of the former hybrid (myosin-linked) (Δ) rather than the latter (actin-linked) (□). The Ca and Sr requirements of the actin-linked regulatory system was not found in squid myosin B, and it seems that the myosin-linked regulatory system is more closely associated with squid myosin B. It should be noted that the Sr requirement of squid crude actin/rabbit myosin (actin-linked) (□) was identical with that of rabbit myosin B (○), which is actin-linked. It is, therefore, likely that a myosin-linked regulatory system is predominant in squid myosin B.

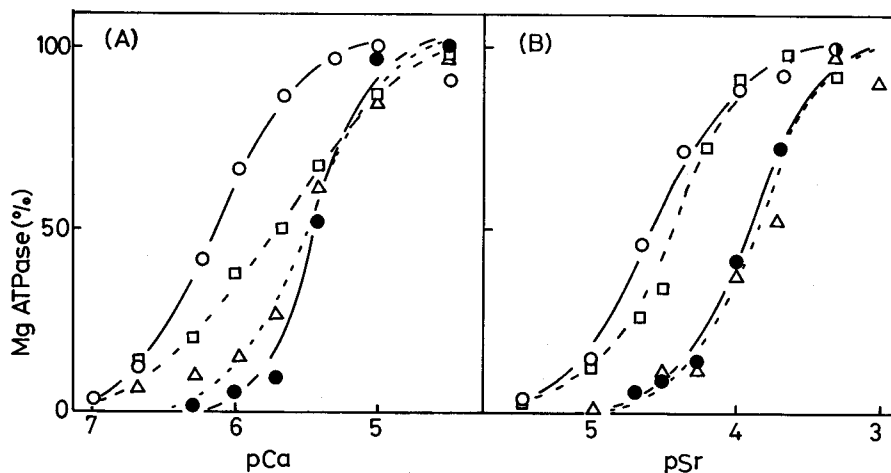


Fig. 18. Mg-ATPase activity of squid myosin B and reconstituted actomyosin as a function of calcium ion or strontium ion concentration.

(●), Squid myosin B (95 $\mu\text{g/ml}$); (Δ), squid myosin (110 $\mu\text{g/ml}$) and skeletal actin (49 $\mu\text{g/ml}$); (□), squid crude actin (139 $\mu\text{g/ml}$) and skeletal myosin (93 $\mu\text{g/ml}$); (○), rabbit skeletal myosin B (102 $\mu\text{g/ml}$). The ATPase assay was conducted as described in Table I. The maximal and minimal ATPase activities obtained under the present conditions were scaled as 100 and 0%, respectively.

It should also be pointed out (Fig. 18) that the concentration of calcium or strontium required for Mg-ATPase of squid mantle myosin B is higher than that required for Mg-ATPase of skeletal myosin B: half maximal activation of Mg-ATPase was obtained at 0.8 μM calcium ions or 28 μM strontium ions with rabbit myosin B and 2.5 μM calcium ions or 140 μM strontium ions with squid myosin B.

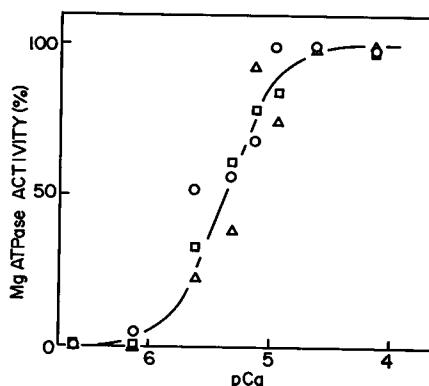
2-B-b Effects of squid and rabbit native tropomyosins on the Ca requirement for Mg-ATPase of squid myosin B

The results described above strongly suggests that myosin-linked Ca regulation is operative in squid myosin B. In order to further establish this, the

author tested whether the addition of large amounts of native tropomyosin to squid myosin B would shift the Ca requirement of myosin B ATPase from myosin-linked to actin-linked or not. As shown in Fig. 19, neither squid native tropomyosin nor rabbit native tropomyosin shift the Ca requirement of squid myosin B.

Fig. 19. Effect of native tropomyosin from squid or rabbit on the Ca requirement of Mg-ATPase of squid myosin B.

ATPase assay was conducted as described in Fig. 18; (○) squid myosin B alone (108 $\mu\text{g}/\text{ml}$); (Δ) squid myosin B (108 $\mu\text{g}/\text{ml}$) with rabbit native tropomyosin (23 $\mu\text{g}/\text{ml}$); and (\square) squid myosin B (108 $\mu\text{g}/\text{ml}$) with squid native tropomyosin (30 $\mu\text{g}/\text{ml}$).



2-C Regulatory light chain from squid muscle

The author found that myosin-linked regulation rather than actin-linked regulation predominates in squid myosin B. This fact necessitated a study of the myosin-linked Ca regulation mechanism in squid mantle muscle.

At first, the author attempted to isolate two types of small polypeptide chains (so-called light chains) from squid myosin, which were considered to be the regulatory subunit in myosin-linked regulation, and to study in detail the functional role of the light chains in myosin-linked Ca regulation. For this purpose, scallop adductor myosin must be employed because squid myosin was not desensitized by EDTA-treatment or by any other techniques tried thus far.

2-C-a Isolation of highly purified squid light chains

As reported by KENDRICK-JONES *et al.*³²⁾, squid myosin showed two light chain bands in a SDS gel and a single band in an urea gel, whereas scallop myosin showed a single band in a SDS gel and two bands in an urea gel. These results indicate that squid light chains differ in size but have a similar net charge while scallop light chains differ in net charge but have a similar size. The two types of light chains of squid myosin were designated as LC-1 (17,000 daltons) and LC-2 (15,000 daltons), and those of scallop myosin as EDTA-LC and SH-LC.

Squid light chains were not released from myosin either by EDTA-treatment¹⁰⁾³²⁾ or by DTNB-treatment³⁶⁾ (data not shown), but they were released by 4 M urea¹¹⁾. Chart II outlines the procedures adopted for the isolation of squid light chains (see also "Methods and materials"). The fractions obtained by ammonium sulfate salting-out were examined by SDS gel electrophoresis. Only a very small amount of light chains was precipitated at 50% saturation, therefore the fraction precipitating between 50 and 100% saturation with ammonium sulfate was

collected. The fraction was dialyzed against a solution containing 0.15 M KCl, 20 mM Tris-HCl (pH 8.0) and 0.1 mM DTT, and centrifuged to remove insoluble materials. The supernatant was subjected to gel filtration, using the same solution for dialysis. As shown in Fig. 20, proteins with molecular sizes larger than those of the light chains appeared at around the void volume [(a) in Fig. 20], and light chains in a pure form [see SDS gel electrophoretic pattern (b) in Fig. 20] were retained and thus appeared in a separate fraction.

Various types of chromatography have been reported to be effective in separating the myosin light chains. The method of PERRIE *et al.*¹²⁾ with some modification, was found to be satisfactory in separating LC-1 and LC-2 of squid myosin. As shown in Fig. 21, LC-2 (15,000 daltons) was eluted with approximately 0.22 M KCl, and LC-1 (17,000 daltons) with approximately 0.25 M KCl.

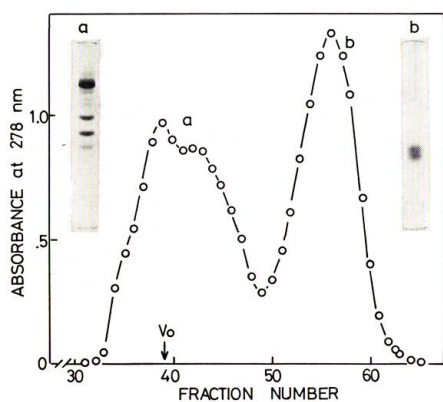


Fig. 20. Sephadex G-75 gel filtration of squid light chains.

Squid light chains were precipitated with ammonium sulfate between 50 and 60% saturation, and the precipitate was applied to a column of Sephadex G-75 (2.5 × 90 cm) in 0.15 M KCl, 20 mM Tris-HCl (pH 8.0), and 0.5 mM DTT. Fractions of 5 ml were collected. The inset photos are of SDS gel electrophoretic patterns of the fractions obtained. SDS gel electrophoresis was conducted as described in Fig. 14.

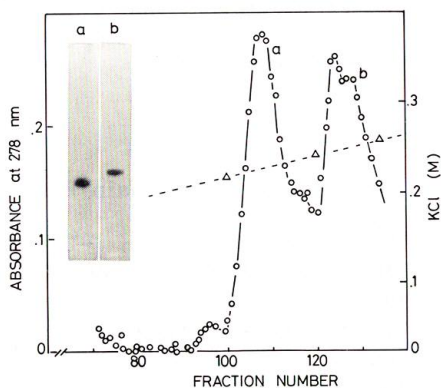


Fig. 21. Separation of light chains of squid myosin by DEAE-Sephadex A25 chromatography.

A solution (approximately 30 mg) of the light chain mixture obtained by Sephadex G-75 gel filtration was applied to a column (1 × 80 cm) of DEAE-Sephadex A25 pre-equilibrated with 0.15 M KCl, 20 mM Tris-HCl (pH 8.0), and 0.5 mM DTT. A linear gradient of KCl, as indicated by the dashed line, was applied from 0.15 M to 0.3 M in 20 mM Tris-HCl (pH 8.0), and 0.5 mM DTT. The flow rate was 10 ml/h, and the volume of each fraction was 5 ml. The SDS gel electrophoretic patterns of the light chain-1 fraction (b) and the light chain-2 fractions (a) are shown in the insets (10% polyacrylamide, 0.1% SDS).

2-C-b *Effect of 2-mercaptoethanol on the patterns of squid light chains in disc gel electrophoresis*

Disc gel electrophoresis was conducted in the presence of various concentrations of 2-mercaptoethanol. Figure 22A shows that the squid LC-2 preparation gave a single band which showed no change in mobility at any of the concentrations of 2-mercaptoethanol tested. In contrast, Fig. 22B shows that the squid LC-1 preparation gave three bands (one major and two minor) in the absence of 2-mercaptoethanol, two bands in the presence of 0.25% 2-mercaptoethanol, and finally a single band when the concentration of 2-mercaptoethanol was increased to 10 to 25%. It seems therefore reasonable to conclude that squid LC-1 is equivalent to scallop SH-LC.

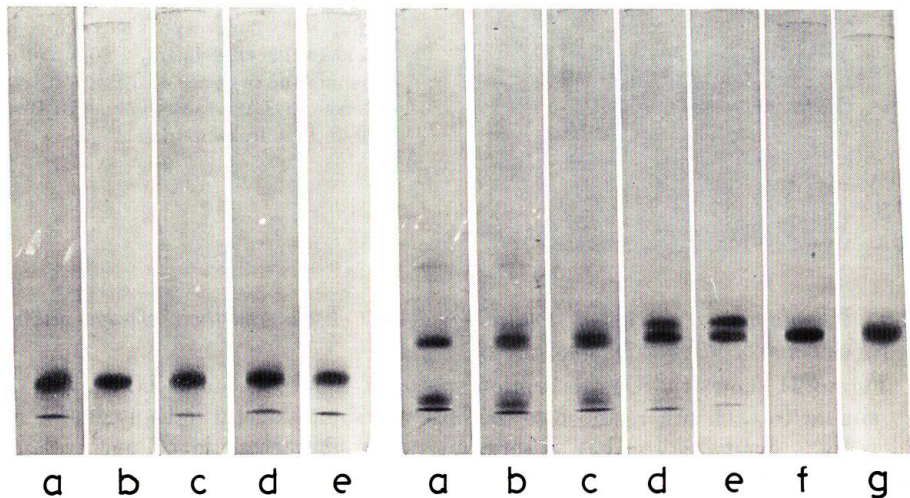


Fig. 22. Effect of 2-mercaptoethanol on the patterns of squid light chains in disc gel electrophoresis.

Disc gel electrophoresis was carried out by the method described by DAVIS. A light chain sample was dissolved in 0.1 M Tris-HCl (pH 8.9) and various concentrations of 2-mercaptoethanol. It was applied to a gel rod of 7.5% polyacrylamide; a solution of 0.05 M Tris and 0.19 M glycine (pH 9.4) was used in the electrophoretic run. Electrophoresis was performed at 2 mA/rod, and the temperature was maintained at around 10°C by circulating cold water. (A), Squid LC-2; (B), squid LC-1. (a) 0%; (b) 0.0025%; (c) 0.025%; (d) 0.25%; (e) 2.5%; (f) 10%; and (g) 25% 2-mercaptoethanol was present.

2-C-c *Effect of calcium on the patterns of squid light chains in disc gel electrophoresis*

HEAD *et al.*³⁷⁾ studied the calcium-binding abilities of troponin-C preparations from various sources by conducting urea gel electrophoresis in the presence of calcium (1 mM CaCl_2) and in its absence (5 mM EGTA). The author found that in urea gel electrophoresis, calcium had no effect on the mobility of either LC-1 or LC-2.

On the other hand, as shown in Fig. 23, the author found that in disc gel electrophoresis, calcium caused a decrease in the mobility of LC-2 but no change in that of LC-1. It is therefore suggested that squid LC-2 can bind calcium. This view is supported, as described in the next section, by the finding that calcium can induce changes in UV absorption spectrum of squid LC-2.

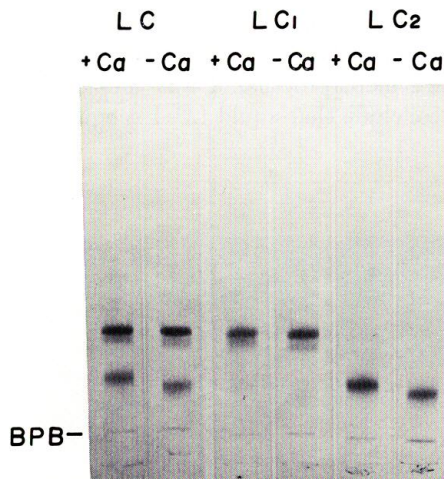


Fig. 23. Calcium-induced change in the mobility of squid light chain in disc gel electrophoresis.

Disc gel electrophoresis was carried out as described in Fig. 22, except that 10% 2-mercaptoethanol was present in this experiment. +Ca and -Ca denote the presence of 1 mM CaCl_2 and 1 mM EGTA, respectively. BPB was used as a front marker.

2-C-d UV absorption spectra of isolated squid light chains and their changes induced by calcium or strontium ions

Figure 24 shows the UV absorption spectra of squid LC-1 and LC-2; they are very similar to each other, and also to those of rabbit skeletal light chains³⁸⁾, and scallop light chains²²⁾, in that absorption peaks characteristic of phenylalanine residues appear.

Figure 25 shows that 1 mM CaCl_2 induced practically no change in the UV absorption spectrum of squid LC-1, but a large change in that of squid LC-2, indicating that calcium does not bind to LC-1 but binds to LC-2. Three positive peaks and three negative peaks are evident, the largest negative changes occurring at 287 nm and at 293 nm (tryptophan residue).

The change at 293 nm was measured as a function of calcium or strontium ion concentration, and the results are shown in Fig. 26. The half-maximal change was obtained with 6.6 μM free calcium ions or with 600 μM free strontium ions. These values are somewhat larger than those obtained previously¹⁰⁾ for the half-maximal activation of actomyosin ATPase: 2 μM free calcium and 140 μM free strontium.

2-C-e UV absorption spectrum of scallop EDTA-LC and the change induced by calcium or strontium ions

It was also found that divalent cations induce a spectral change of scallop EDTA-LC (Fig. 27). Figure 27A shows the UV absorption spectrum of scallop EDTA-LC, which is identical with that reported by KENDRICK-JONES *et al.*⁴⁾

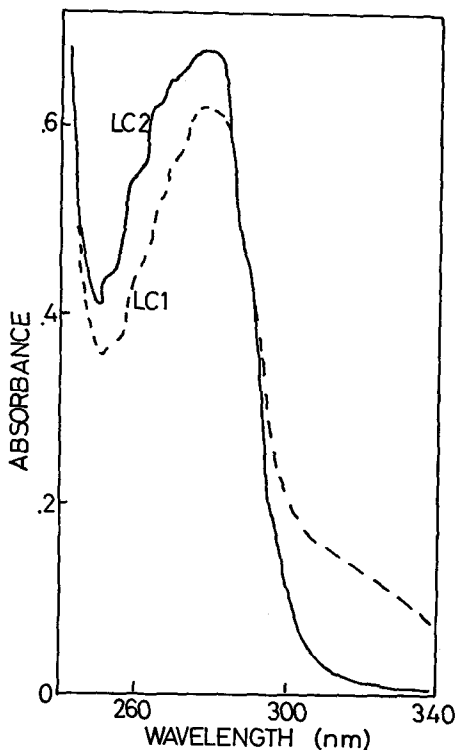


Fig. 24. Ultraviolet absorption spectra of isolated squid light chains.

UV absorption spectra of isolated light chains were measured in 0.1 M KCl, and 20 mM histidine-KOH (pH 6.8). ----, light chain-1 (0.98 mg/ml); —, light chain-2 (1.15 mg/ml). $E_{280\text{nm}}^{1\%} = 5.4$ for LC-1, and 6.9 for LC-2.

the calcium concentration required for half-maximal activation of scallop myosin B ATPase was $2.5 \mu\text{M}$ free calcium ions, whereas that required for the half-maximal change in the induced spectral change was $320 \mu\text{M}$ free calcium ions; the strontium concentration required for half-maximal activation of myosin B ATPase was $100 \mu\text{M}$ free strontium, whereas that required for the half-maximal change in the induced spectral change was 4.0 mM free strontium ions.

2-D Desensitization and resensitization of scallop myosin

2-D-a Desensitization of scallop myosin by EDTA-treatment

As reported previously¹⁰), EDTA-treatment did not remove any light chains from squid myosin and did not reduce the Ca sensitivity of acto-squid myosin

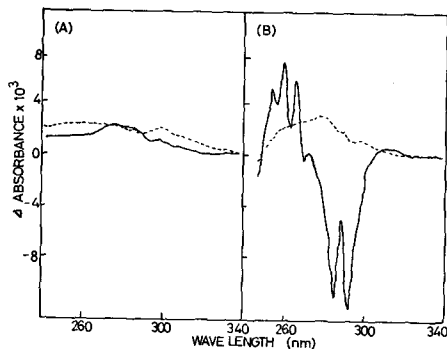


Fig. 25. Calcium-induced spectral change of isolated squid light chains.

The absorption difference spectra were measured in 0.1 M KCl, and 20 mM histidine-KOH (pH 6.9). 1 mM CaCl_2 was present in the sample cell, and 1 mM EGTA in reference cell. Base lines are indicated by dashed lines. (A) Light chain-1 (0.98 mg/ml), and (B) light chain-2 (1.15 mg/ml).

Figure 27B shows that 1 mM CaCl_2 induced a change in the UV absorption spectrum of scallop EDTA-LC; it is different from that of squid LC-2 in that it lacks the negative change at 293 nm (tryptophan residue).

However, as shown in Fig. 28, the divalent cation concentrations required to induce the spectral changes were too high for this process to account for the sensitivity of scallop myosin B ATPase to divalent cations. The

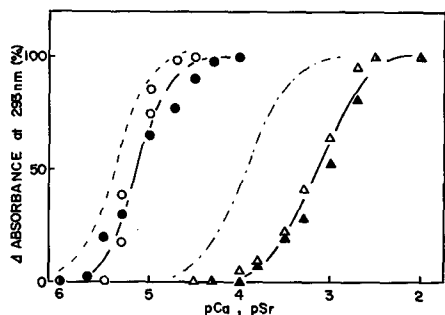


Fig. 26. Calcium and strontium binding to squid light chain-2.

The relative magnitude of the difference spectrum at 293 nm was plotted as a function of the free calcium or strontium ion concentration. The concentrations of light chain-2 employed were the same as in Fig. 25. The concentrations of free calcium and strontium ions were maintained by using Ca-EGTA and Sr-EGTA buffers. Stability constants of $5 \times 10^5 \text{ M}^{-1}$ and $2 \times 10^2 \text{ M}^{-1}$ were used for the Ca-EGTA and Sr-EGTA complexes, respectively. The reference cell always contained 1 mM EGTA. Open and closed symbols represent different light chain-2 preparations. (●, ○) Calcium-induced difference spectra; (▲, △) strontium-induced difference spectra. The Ca requirement (----) and Sr requirement (—) of skeletal acto-squid myosin ATPase activity are also shown.

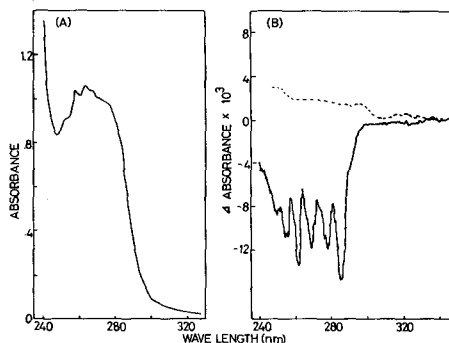
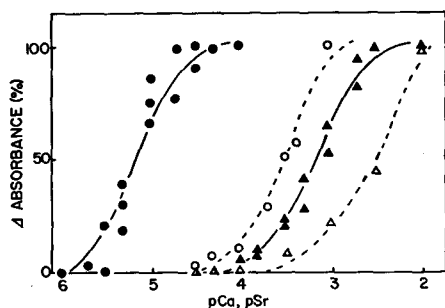


Fig. 27. Ultraviolet absorption spectrum and calcium-induced spectral change of scallop EDTA-light chain.

(A) The UV absorption spectrum of EDTA-light chain (2.2 mg/ml) was obtained in a medium containing 0.1 M KCl, 20 mM histidine-KOH (pH 6.8). (B) The calcium-induced spectral change of EDTA-light chain was obtained in the medium described above, except that the sample cell also contained 1 mM CaCl_2 and the reference cell 1 mM EGTA. The dashed line indicates the base line.

Fig. 28. The calcium and strontium requirements for producing difference spectra in scallop EDTA-light chain and for activation of scallop myosin B ATPase.

The relative magnitude of the difference spectrum at 287 nm was plotted as a function of the free calcium or strontium concentration. The experimental conditions were as described in Fig. 25. The Mg-ATPase activities of scallop myosin B were measured at 25°C in a medium containing 30 mM KCl, 20 mM Tris-maleate (pH 6.8), 2 mM MgCl_2 , 1 mM ATP, and various concentrations of free calcium or strontium ions. (○) Calcium-induced difference spectrum; (△) strontium-induced difference spectrum; (●) Ca requirement for Mg-ATPase; and (▲) Sr requirement for Mg-ATPase.

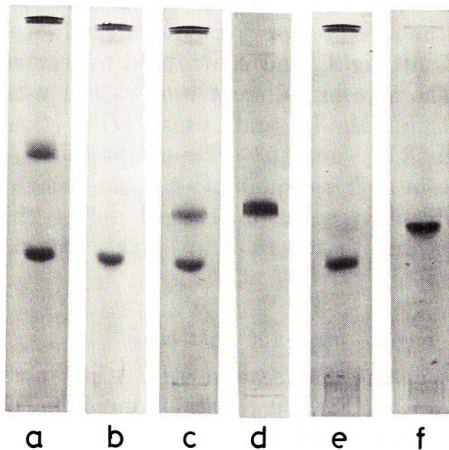


Fig. 29. Binding of squid light chains to scallop desensitized myosin.

Scallop desensitized myosin was prepared by EDTA-treatment as described in Fig. 31. Squid light chains were added to scallop desensitized myosin in 0.5 M KCl, 20 mM Tris-maleate (pH 7.5), and 2 mM $MgCl_2$. Ten volumes of 1 mM $MgCl_2$ were then added, and the mixture was centrifuged. Unbound light chains in the supernatant were removed. The myosin pellet was dissolved in a 8 M urea, 0.1 M Tris-HCl (pH 8.9), and 5% 2-mercaptoethanol, and then applied to a 7.5% polyacrylamide gel rod containing 8 M urea and 0.375 M Tris-HCl (pH 8.9). Urea-gel electrophoresis was carried out by a modification of the method of PERRIE *et al.*¹²⁾ The running buffer contained 0.05 M Tris and 0.19 M glycine (pH 9.4). Approximately 50 μ g of protein was loaded on each gel. (a) Untreated scallop myosin, (b) desensitized scallop myosin, (c) scallop desensitized myosin incubated with squid LC-2, (d) squid LC-2, (e) scallop desensitized myosin incubated with squid LC-1, and (f) squid LC-1.

2-D-b Binding of squid light chains to scallop desensitized myosin

The abilities of squid LC-1 and of squid LC-2 to bind to desensitized (EDTA-treated) scallop myosin [(b) in Fig. 29] were tested in the following way (see also "Methods and materials"): 0.6 mg/ml of LC-1 or LC-2 was mixed with 6 mg/ml of

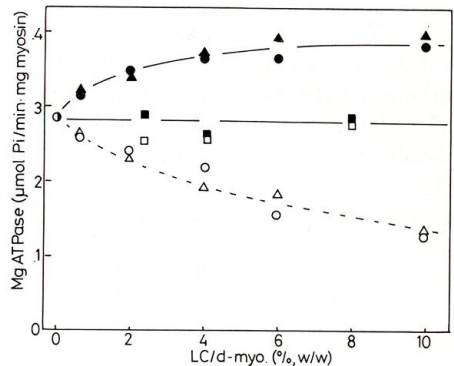


Fig. 30. Resensitization of scallop desensitized myosin by squid light chains and by scallop EDTA-light chain.

Various amounts of squid light chains or scallop EDTA-light chain were added to scallop desensitized myosin in the medium described in Fig. 29. Mg-ATPase activities of the desensitized myosin were assayed at 25°C in the presence of skeletal F-actin in a medium containing 30 mM KCl, 20 mM Tris-maleate (pH 6.8), 2 mM $MgCl_2$, 1 mM ATP, and either 0.1 mM $CaCl_2$ (closed) or 0.5 mM EGTA (open). 100–110 μ g/ml of desensitized myosin and 35 μ g/ml of skeletal F-actin were used. (●, ○) squid LC-2, (■, □) scallop EDTA-LC.

ATPase. Thus, desensitized scallop myosin was used to study the functional role of squid light chains.

EDTA-treatment of scallop myosin was repeated five times at 0–4°C; as shown in Fig. 29, EDTA-LC were selectively and completely removed from scallop myosin.

desensitized scallop myosin in a solution containing 0.5 M KCl, 20 mM Trismaleate (pH 7.5), 2 mM MgCl₂, and 1 mM DTT, and the mixture was kept overnight in ice-water. It was then diluted with 10 vols of 1 mM MgCl₂, and centrifuged to remove unbound light chains in the supernatant. The myosin sediment was washed with 0.1 M KCl, 20 mM Tris-maleate (pH 7.5), 1 mM MgCl₂, and 1 mM DTT, then dissolved in 8 M urea, 0.1 M Tris-HCl (pH 8.9), and 5% 2-mercaptoethanol. Figure 29 shows that desensitized scallop myosin took up squid LC-2 in an amount approximately equal to its own SH-LC, which remained unchanged during the desensitization and resensitization procedures, and that it took up practically no squid LC-1 (see also Fig. 32).

2-D-c Resensitization of scallop desensitized myosin by squid light chain

The resensitizing effects of squid light chains and scallop EDTA-LC were examined, and the results obtained are shown in Fig. 30. Various amounts of scallop EDTA-LC, squid LC-1 or squid LC-2 were added to desensitized scallop myosin in the presence of magnesium ion, and then combined with rabbit skeletal actin in a weight ratio of 1/3 for actin/myosin. The Mg-ATPase activity of the reconstituted actomyosin was measured in the presence and absence of calcium. Mg-ATPase of acto-desensitized myosin showed no Ca sensitivity. Squid LC-1, which did not bind to desensitized scallop myosin (Fig. 29), had no effect on the ATPase activity either in the presence or absence of calcium. On the other hand, squid LC-2 increased the activity in the presence of calcium and decreased it in its absence, thus conferring Ca sensitivity on acto-desensitized myosin. Moreover, the resensitizing effect of squid LC-2 was quantitatively equal to that of scallop EDTA-LC.

These results with scallop myosin are somewhat different from those reported by SZENT-GYÖRGYI *et al.*⁴⁾ For instance, they reported that only one mol of EDTA-LC can readily be removed by EDTA-treatment, with complete loss of Ca sensitivity, and that removal of the remaining EDTA-LC requires DTNB-treatment, resulting in incomplete resensitization. Based mainly on these results, they proposed that cooperation between either two head (S-1) of scallop myosin or two EDTA-LC is involved in the molecular mechanism of Ca regulation in scallop adductor muscle. However, the author found (Fig. 29) that EDTA-treatment removes all the EDTA-LC from scallop adductor myosin. The author therefore conducted further studies on the desensitization and resensitization of scallop adductor myosin. Removal of EDTA-LC was evaluated by measuring the staining density in urea gel electrophoretic patterns, and Ca sensitivity was determined by measuring Mg-ATPase activities in the presence and absence of calcium. Since SZENT-GYÖRGYI *et al.* stated that the reversibility of desensitization-resensitization is better with myofibrils than myosin alone, the author also carried out EDTA-treatment with myofibrils. It was found (Fig. 31) that EDTA-treatment removed all the EDTA-LC from scallop myosin or myofibrils. Moreover, Fig. 31 shows that scallop desensitized myosin remaining one mol of EDTA-LC per mol of myosin also retained a definite Ca sensitivity, and that removal of not half the total amount of EDTA-LC but of all it is required to achieve a complete loss of Ca sensitivity.

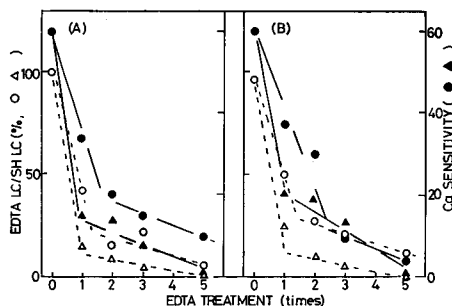


Fig. 31. EDTA-treatment of scallop myosin and myofibrils.

Scallop myosin (A) and myofibrils (B) were suspended in 10 mM EDTA, 30 mM KCl, and 10 mM Tris-HCl (pH 7.5) for 15 min at 0–4°C or at 20°C, then centrifuged at 6,000 \times g for 10 min, thus obtaining the sediments. This procedure was repeated 5 times. The contents of EDTA-LC in myosin and myofibrils were determined by urea-gel electrophoresis, and expressed as the staining density ratio of EDTA-LC band to SH-LC band. The Ca sensitivity was expressed in terms of Mg-ATPase activity and is expressed as $(1 - A_{EG}/A_{Ca}) \times 100$, where A_{EG} and A_{Ca} are the activity in the presence of EGTA and in the presence of $CaCl_2$, respectively. (A) Scallop myosin; (B) scallop myofibrils; (●, ○) EDTA-treatment at 0–4°C; (▲, △) EDTA-treatment at 20°C; (●, ▲) Ca sensitivity; and (○, △) EDTA-LC content.

2-D-d Relationship between EDTA-LC (or squid LC-2) content of myosin and Ca sensitivity of acto-scallop myosin

Using 5-times-EDTA-treated scallop myosin, which was completely free from EDTA-LC and from Ca sensitivity, we studied its recombination with isolated scallop EDTA-LC, squid LC-1 and LC-2 as well as its resensitization in terms of the Mg-ATPase activity of reconstituted actomyosin. The results obtained are shown in Fig. 32. The amount of light chains bound to scallop desensitized myosin was expressed as the ratio of the Fast Green staining density of the recombined light chain band to that of the SH-LC band retained in desensitized myosin. The symbol (●) in Fig. 32A indicates the density ratio of EDTA-LC to SH-LC in untreated scallop myosin. The Ca sensitivity was expressed in terms of $(1 - A_E/A_{Ca}) \times 100$, where A_E and A_{Ca} are the ATPase activity in the presence of EGTA and in the presence of calcium, respectively. The symbol (⊙) in Fig. 32B indicates the Ca sensitivity of untreated scallop myosin.

Dashed lines in Fig. 32 indicate that both the recombination and resensitization were saturated when the amount of either EDTA-LC or squid LC-2 added reached approximately 7.5% weight ratio to scallop desensitized myosin, where the amount of the light chains bound was practically equal to that of EDTA-LC present in untreated scallop myosin. If one assumes that one mol of scallop myosin (480,000 daltons) is composed of two mol of heavy chains ($2 \times 206,000$ daltons), two mol of EDTA-LC ($2 \times 17,000$ daltons), and two mol of SH-LC ($2 \times 17,000$ daltons), then the weight ratio of EDTA-LC to desensitized myosin is expected to be $17/(206+17) = 0.076$. The numerical agreement between the expected value and the observed value is remarkably good. It should also be noted that a small amount of

squid LC-1 preparation can bind to desensitized myosin but does not confer any Ca sensitivity on desensitized myosin. The results shown in Fig. 32 indicate that the restoration of Ca sensitivity was proportional to the amount of scallop EDTA-LC or squid LC-2 bound to scallop desensitized myosin. It therefore appears that cooperation of two mol of EDTA-LC in scallop myosin is not involved in myosin-linked Ca regulation⁴).

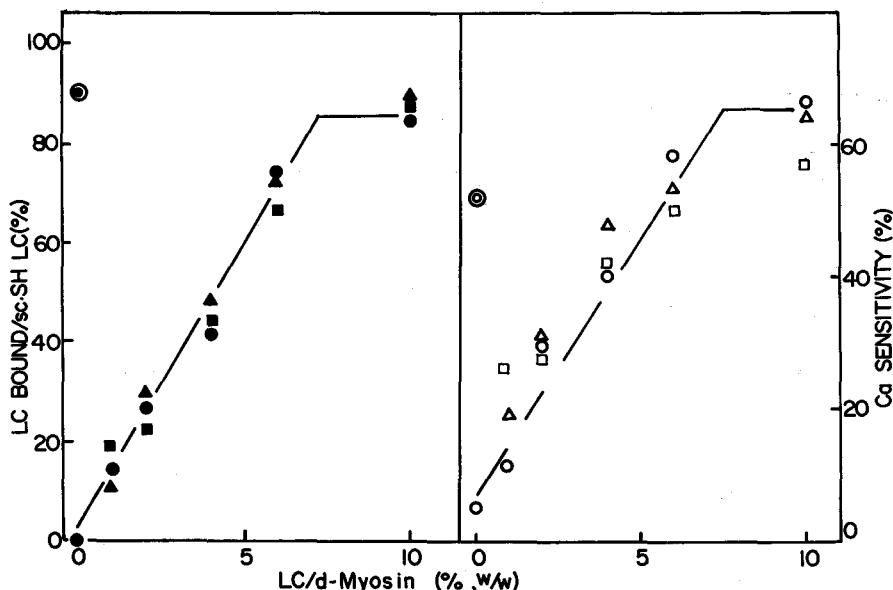


Fig. 32. Recombination and resensitization of scallop desensitized myosin.

(A) Recombination of light chains with scallop desensitized myosin was determined by urea-gel electrophoresis. The amount of light chains bound to desensitized myosin was determined by densitometry at 640 nm with Fast Green staining, and is expressed in terms of (light chain bound)/(scallop SH-LC) \times 100. (\times) Squid light chain-1; (\bullet) squid light chain-2; (\blacksquare) scallop EDTA-LC; and (\blacktriangle) a mixture of squid light chain-2 and scallop EDTA-LC (1/1, w/w). (\odot) The EDTA-LC content in untreated scallop myosin. (B) Resensitization of scallop desensitized myosin by squid light chains or by scallop EDTA-LC was studied by measuring the Mg-ATPase activities in combination with skeletal F-actin. The recovery of Ca sensitivity was estimated by measuring Mg-ATPase activities (see Figs. 31 and 29). (\times) Squid light chain-1; (\circ) squid light chain-2; (\square) scallop EDTA-LC; and (\triangle) a mixture of squid light chain-2 and scallop EDTA-LC (1/1, w/w). (\odot) Ca sensitivity of untreated myosin.

2-D-e Competitive binding of EDTA-LC and squid LC-2 to scallop desensitized myosin

The recombination and resensitization were also studied by adding a mixture of scallop EDTA-LC and squid LC-2 in various weight ratios, while the total amount of light chains added was kept at 10% (w/w) of desensitized myosin. As shown in Fig. 33, as the ratio of squid LC-2 to EDTA-LC increased in the

mixture added, the amount of EDTA-LC bound decreased and that of LC-2 increased, thus indicating competitive binding between EDTA-LC and squid LC-2. Meanwhile, the total amount of light chains bound (EDTA-LC+squid LC-2), as well as the Ca sensitivity, remained nearly unchanged. One can therefore conclude that squid LC-2 is functionally identical with scallop EDTA-LC, and that scallop desensitized myosin free from EDTA-LC retains its original ability to bind light chains and to respond to calcium ions.

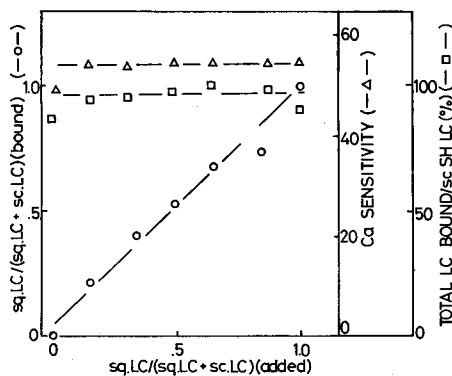


Fig. 33. Competitive binding of squid light chain-2 and scallop EDTA-LC to scallop desensitized myosin.

A mixture of squid light chain-2 and scallop EDTA-LC in various ratios (the total amount of light chains added was kept at 10% of myosin by weight) was added to scallop desensitized myosin in the medium described in Fig. 30. The amount of light chains bound was determined by urea-gel electrophoresis (see Fig. 32). The amount of squid light chain-2 bound was expressed as a ratio to the total amount of light chain bound (○). The total amount of light chain bound was also expressed as its ratio to the amount of scallop SH-LC (□). The recovery of Ca sensitivity (△) was estimated by measuring Mg-ATPase activities (see Figs. 29 and 31).

2-E Calcium binding of myosin and of regulatory light chains

2-E-a Calcium binding of isolated squid LC-2 and scallop EDTA-LC

The effects of calcium and strontium ions on the UV absorption spectra of light chain subunits isolated from squid myosin and from scallop adductor myosin were studied. The author found (a) that the divalent cations induced difference spectra in regulatory light chains (squid LC-2 and scallop EDTA-LC) but not in the second type of light chains (LC-1 and SH-LC), and (b) that the strontium and calcium concentrations required for inducing difference spectra were much higher than those required for activation of the Mg-ATPase of myosin B. In other words, the results obtained were qualitatively in favor of, but quantitatively inconsistent with, the possibility that the divalent cation-induced change in the regulatory light chain conformation may be the first step in a series of reactions, which lead to "contraction" of actomyosin.

In the present study, the author measured calcium binding to regulatory light chains in an isolated form and with myosins (light chains in a bound form).

Isolated regulatory light chains were purified by Sephadex G-75 gel filtration. The molecular size of scallop EDTA-LC estimated by the gel filtration was about 50,000 daltons and that of squid LC-2 was about 20,000 daltons. This suggests that scallop EDTA-LC is in an aggregated form. Calcium binding to regulatory light chain was then measured by using ^{45}Ca in equilibrium dialysis²¹⁾.

As shown in Fig. 34, isolated light chains had two types of calcium binding sites: calcium binding with the first type of site occurred at about 1–10 μM free calcium whereas that with second type occurred at about 100–1,000 μM free calcium. The amount of calcium bound to the first type of site was small: approximately 0.4 mol of calcium per mol of squid LC-2 (15,000 daltons) and 0.15 mol of calcium per mol of scallop EDTA-LC (17,000 daltons). Moreover, the amount of calcium bound was greatly reduced when 2.5 mM MgCl_2 was present.

A lower calcium binding ability of isolated scallop EDTA-LC was also reported by SZENT-GYÖRGYI *et al.*⁴⁾. In the present study, the author found that squid regulatory light chain retained its calcium binding ability even if it was released from the heavy chain. In this respect, squid regulatory light chain is clearly different from the EDTA-LC of scallop.

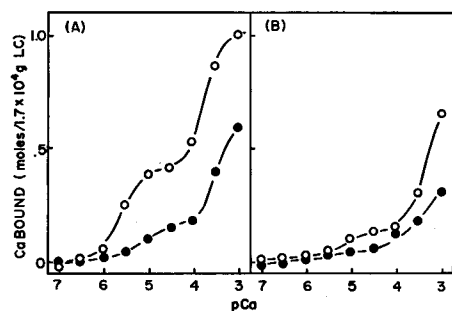


Fig. 34. Calcium binding to isolated regulatory light chains from squid and scallop muscles.

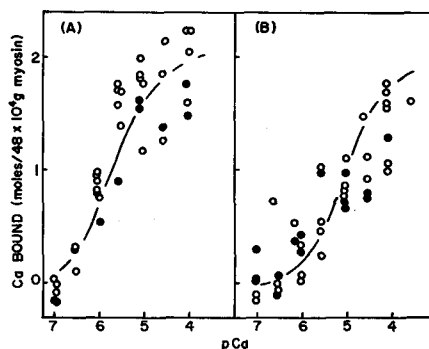
Calcium binding was measured at 20°C by using ^{45}Ca in equilibrium dialysis in a medium containing 0.1 M KCl, 20 mM histidine (pH 6.8), and no (o) or 2.5 mM MgCl_2 (●). Free calcium concentration was set by using a Ca-EGTA buffer system. (A) Squid LC-2, (B) scallop EDTA-LC.

2-E-b Calcium binding of squid and scallop myosin

On the other hand, as shown in Fig. 35, calcium binding to myosins was very different from that to isolated light chains. The amount of calcium bound to myosin was about two mol per mol of myosin (4.8×10^5 daltons), which is equivalent to one mol of calcium per mol of light chain (in a bound form). Only one type of binding site was detectable, and the the half-maximal binding occurred at about 2.0 μM free calcium with squid myosin, and at about 7.9 μM free calcium with scallop myosin. These concentrations of free calcium ions were about equal to those required for activation of the ATPase of acto-squid myosin and for that of acto-scallop myosin (see Figs. 37 and 50A). Therefore, the difference between the calcium concentration required for activating myosin B ATPase and that required for inducing difference spectra of isolated light chains may be understood as the difference in calcium binding between a bound form of light chains and an isolated form of light chains (see Figs. 34 and 35).

Fig. 35. Calcium binding to myosin from squid and scallop muscles.

Calcium binding was measured under the conditions described in Fig. 34, except that 0.3 M KCl was used instead of 0.1 M KCl. The same symbols as in Fig. 34 are used. (A) Squid myosin, (B) scallop myosin.



2-E-c *Effect of desensitization and resensitization on the calcium binding of scallop myosin*

Moreover, it was also shown (Fig. 36) that the regulatory light chain is responsible for the calcium binding described above. The author studied the effect of desensitization of myosin on its calcium binding ability. Figure 36 shows that desensitized scallop myosin, which was completely free of EDTA-LC, lost its calcium binding ability (A), and that the lost ability was fully recovered by adding either scallop EDTA-LC(C) or squid LC-2(B) to desensitized scallop myosin

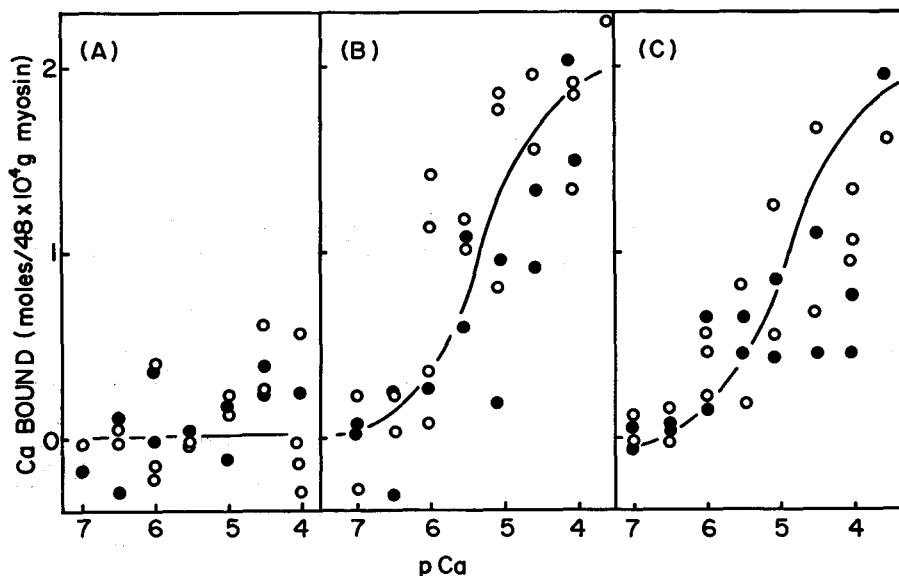


Fig. 36. Calcium binding to desensitized and resensitized scallop myosins.

Calcium binding was measured under the same conditions as in Fig. 35. Symbols are the same as in Fig. 35. (A) Desensitized scallop myosin; (B) resensitized scallop myosin with squid LC-2; and (C) resensitized scallop myosin with scallop EDTA-LC.

(approximately two mol of light chain per mol of myosin). It should be pointed out that calcium binding to resensitized myosin, like that to untreated myosin (see Fig. 35), was unaffected by the addition of 2.5 mM $MgCl_2$.

It was found that scallop myosin and scallop resensitized myosin were capable of binding approximately two mol of calcium per mol of myosin even if $MgCl_2$ was absent. Moreover, EDTA-LC in an isolated form was found to have little calcium binding ability at around 10 μM calcium ions (see Fig. 34). Therefore, these results suggest tht EDTA-LC was present in a form bound to myosin even if $MgCl_2$ was absent. This view is further supported by the recent finding of OJIMA *et al.* (personal communication) that the release of EDTA-LC from Akazara scallop myosin was prevented by the presence of small amounts of calcium even if $MgCl_2$ was absent.

2-F Calcium sensitivity of squid myosin alone

In the previous sections, Ca sensitivity of acto-squid myosin or acto-scallop myosin were studied. Recently, ASADA *et al.*³⁹⁾ showed that Mg-ATPase of clam foot myosin alone was sensitive to calcium ion. In this section, therefore, the Ca sensitivity of myosin alone from squid and scallop was studied.

2-F-a Ca requirement for activation of Mg-ATPase of squid myosin alone

As shown in Fig. 37, Mg-ATPase of squid myosin alone was activated by a very low concentration of calcium ions (1–10 μM) and half-maximal activation was

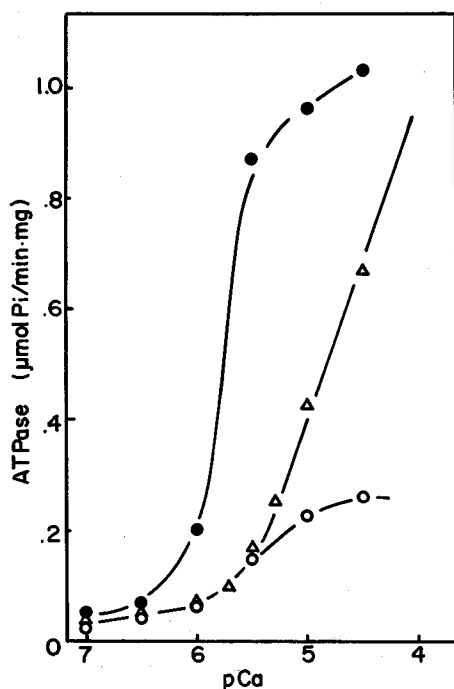


Fig. 37. Calcium requirements for Mg-ATPase activity of squid myosin and of acto-squid myosin and for Ca-ATPase activity of squid myosin alone.

Mg-ATPase of myosin alone (○) and of acto-myosin (●) were assayed in a medium containing 30 mM KCl, 20 mM Tris-maleate (pH 6.8), 2 mM $MgCl_2$ and 1 mM ATP at 25°C. Ca-ATPase of myosin alone (▲) was assayed in the above medium without 2 mM $MgCl_2$. Free calcium concentration was set by using a Ca-EGTA buffer system.

obtained at $3 \mu\text{M}$ free calcium ion, which was identical with that obtained in the activation of Mg-ATPase of acto-squid myosin. These results indicated that squid myosin Mg-ATPase like actomyosin Mg-ATPase was sensitive to calcium ion. It should be pointed out that myosin Mg-ATPase activity was remarkably activated by F-actin in the presence of calcium. In addition, Ca-ATPase activity of squid myosin (activity in the absence of MgCl_2) was able to be distinguished from Mg-ATPase activity by the concentration of calcium required for half-maximal activation; $3 \mu\text{M}$ calcium for Mg-ATPase and $150 \mu\text{M}$ for Ca-ATPase.

2-F-b Effects of temperature and pH on Ca sensitivity

As shown in Fig. 38, a change in the temperature of the ATPase assay from 5°C to 30°C did not affect the Ca sensitivity of squid myosin, that is, Ca sensitivity was observed at all temperatures tested.

Ca sensitivity of squid myosin also detectable even if the pH for the ATPase was changed from 6.0 to 9.5 (Fig. 39).

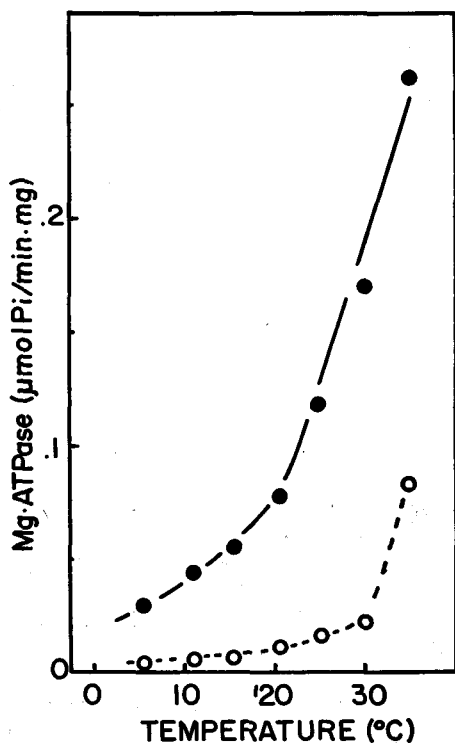


Fig. 38. Temperature dependence of Mg-ATPase activity of squid myosin.

Mg-ATPase activity of squid myosin alone was measured in a medium containing 30 mM KCl, 20 mM Tris-maleate (pH 6.8), 2 mM MgCl_2 , 1 mM ATP and either 0.1 mM CaCl_2 (●) or 0.5 mM EGTA (○). Reaction temperature was changed from 5°C to 30°C .

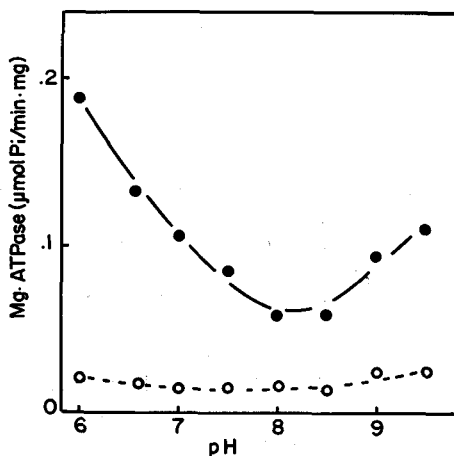


Fig. 39. pH dependence of Mg-ATPase activity of squid myosin.

Mg-ATPase of squid myosin alone was assayed in a medium containing 30 mM KCl, 2 mM MgCl_2 , 1 mM ATP and either 0.1 mM CaCl_2 (●) or 0.5 mM EGTA (○). pH for ATPase assay was changed from 6.0 to 9.5 using 20 mM Tris-maleate or a Tris-HCl buffer system.

2-F-c *Effect of KCl concentration on Ca sensitivity of squid myosin*

A change in KCl concentration for the ATPase assay remarkably affected the Ca sensitivity of squid myosin alone. As shown in Fig. 40, Mg-ATPase of squid myosin alone showed maximal activity at around 0.3 M KCl in the presence of calcium. The activity in the absence of calcium was considerably inhibited in low-salt medium (lower than 0.2 M), but it was gradually increased with increasing KCl concentration, reaching the same activity as that in the presence of calcium at 0.4 M KCl. In other word, the Ca sensitivity of Mg-ATPase activity of squid myosin alone was observed only when the KCl concentration was lower than 0.4 M. On the other hand, actin-activation of Mg-ATPase of myosin occurred when the KCl concentration was lower than 0.2 M. Figure 40B shows that in accordance with the actin-activated Mg-ATPase in Fig. 40A, superprecipitation of acto-squid myosin occurred only when the KCl concentration was lower than 0.2 M. Therefore, it is possible to distinguish the effect of KCl on actin-activation from that on Ca sensitivity of myosin alone.

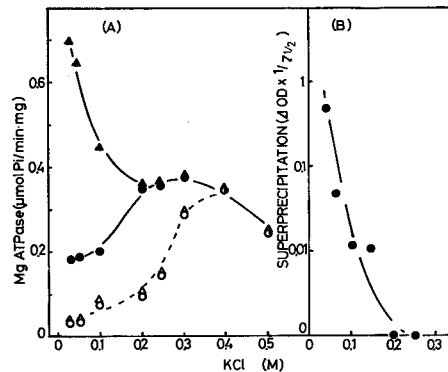


Fig. 40. KCl concentration dependence of Mg-ATPase and that of superprecipitation of acto-squid myosin.

(A) The ATPase activity of myosin (●, ○) and that of acto-squid myosin (▲, Δ) were assayed at 25°C in a medium containing 20 mM Tris-maleate (pH 6.8), 2 mM MgCl_2 , 1 mM ATP, either 0.1 mM CaCl_2 (closed) or 0.5 mM EGTA (open), an various concentrations of KCl from 0.03 M to 0.5 M. (B) The superprecipitation activity of acto-squid myosin was assayed under the conditions used for the ATPase activity (A), but in the presence of 0.1 mM CaCl_2 .

The effect of KCl on Ca sensitivity was further studied by KCl concentration jump method. Figure 41 shows the time course of Pi liberation by Mg-ATPase of squid myosin alone. When the KCl concentration was rapidly increased from 0.03 M to 0.4 M at the time indicated by the arrows, the immediate increase in Pi liberation in the absence of calcium occurred. Moreover, it was no longer sensitive to calcium. It should be noted that no lag phase in the time course of Pi liberation was found during this treatment. The recovery of Ca sensitivity by dilution of KCl was also tested. As shown in Fig. 42, when the KCl concentration in assay medium was decreased rapidly from 0.4 M to 0.2 M, the Ca sensitivity was recovered with no lag time.

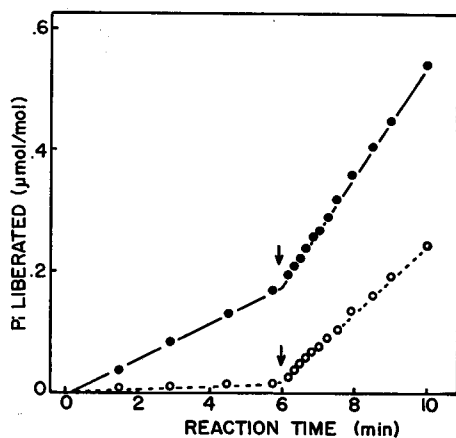
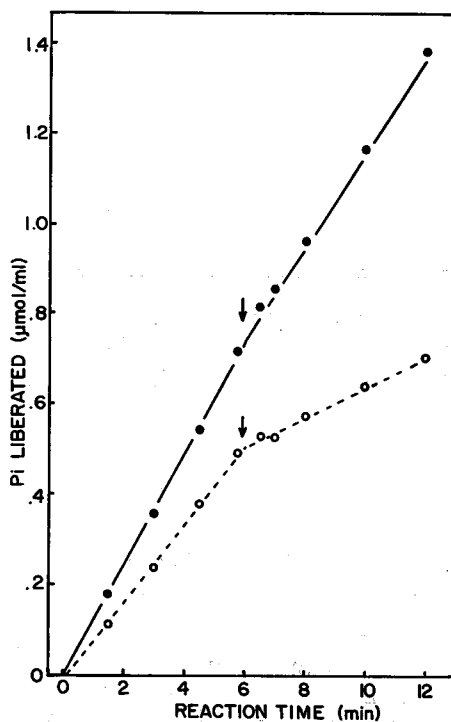


Fig. 41. Effect of KCl concentration on Ca sensitivity of squid myosin.

ATPase of myosin alone was assayed in the medium containing 30 mM KCl, 20 mM Tris-maleate (pH 6.8), 2 mM MgCl₂, 1 mM ATP and either 0.1 mM CaCl₂ (●) or 0.5 mM EGTA (○). At the time indicated by the arrows, the KCl concentration was changed from 0.03 M to 0.4 M without changing other constituents of the medium.

Fig. 42. Recovery of Ca sensitivity of squid myosin by dilution of KCl.

ATPase was assayed in 0.4 M KCl, 20 mM Tris-maleate (pH 6.8), 2 mM MgCl₂, 1 mM ATP and either 0.1 mM CaCl₂ (●) or 0.5 mM EGTA (○). At the time indicated by the arrows, KCl concentration was reduced to 0.2 M without changing other constituents of the medium.



2-F-d ITP as substrate for squid myosin

In order to know whether ATP was essential for Ca sensitivity of myosin as substrate or not, ITP instead of ATP was used in the same experiment of Fig. 42. The KCl concentration dependence of Mg-ITPase of squid myosin alone was shown in Fig. 43. Essentially the same KCl concentration dependence of ITPase

activity was obtained as that of ATPase activity; Mg-ITPase showed Ca sensitivity in a low-salt medium, but lost it in high-salt medium. Therefore, ATP was found not to be essential for Ca sensitivity of myosin.

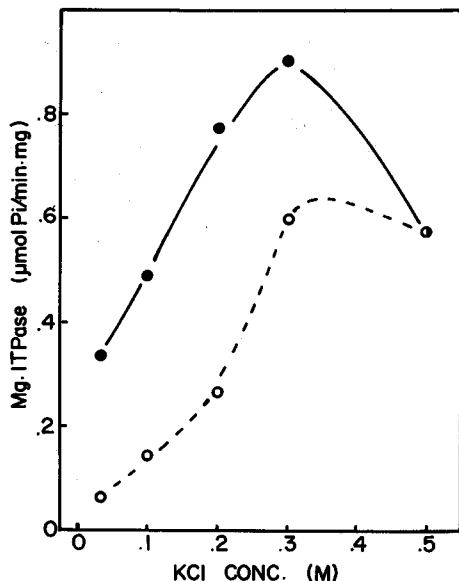


Fig. 43. KCl concentration dependence of Mg-ITPase of squid myosin.

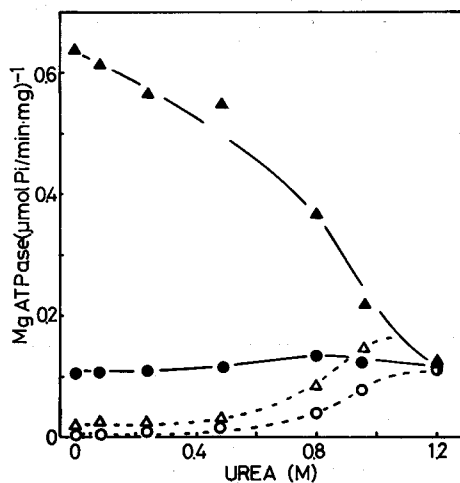
ITPase activity of squid myosin alone was assayed under the same conditions as in Fig. 40A, except that 0.5 mM ITP was used instead of 1 mM ATP. The same symbols are also used.

2-F-e *Effect of urea on Ca sensitivity of squid myosin*

Ca sensitivity of Mg-ATPase of squid myosin alone was measured in a medium containing various concentrations of urea. As shown in Fig. 44, Mg-ATPase of myosin alone was Ca sensitive when the urea concentration was lower than 1M, it

Fig. 44. Effect of urea on Mg-ATPase activities of squid myosin and of actomyosin.

The ATPase activity of myosin alone (●, ○) and that of actomyosin (▲, △) were assayed under the same conditions as in Fig. 38, except that various concentrations of urea from 0 M to 1.2 M were present in the reaction mixture.



was lost in the presence of 1.2 M urea. It should be pointed out that the Mg-ATPase activity of myosin alone in the presence of calcium was unaffected by urea. The effect of urea on Ca sensitivity of myosin alone and on actin-activation of Mg-ATPase were then compared. Figure 44 showed that actin-activation of myosin Mg-ATPase in the presence of calcium decreased as the urea concentration was increased, though it still occurred even at 1 M urea. On the other hand, actin-activation in the absence of calcium increased slightly at concentrations of urea higher than 1 M. The decrease in actin-activation of myosin Mg-ATPase in the presence of calcium was closely related to the irregular conformation of myosin thick-filaments induced by urea⁴⁰. It should be noted that at the same concentration of urea (1 M), both myosin Mg-ATPase and the actin-activation of myosin ATPase increased in the absence of calcium.

Therefore, the following conclusions were suggested: when the inhibitory effect of regulatory light chain on myosin ATPase was removed by urea, the inhibitory effect on the interaction between actin and myosin was also removed, and this resulted in an increase of actin-activation in the absence of calcium.

The effect of urea on the Ca sensitivity of myosin Mg-ATPase was further studied by urea concentration jump method. As shown in Fig. 45, when urea was added to the reaction mixture to a final concentration of 1.1 M, at the time indicated by the arrow, Pi liberation in the absence of calcium immediately increased with no lag time. Then the reversibility of the effect of urea on Ca sensitivity of myosin was tested. The result was shown in Fig. 46. When the

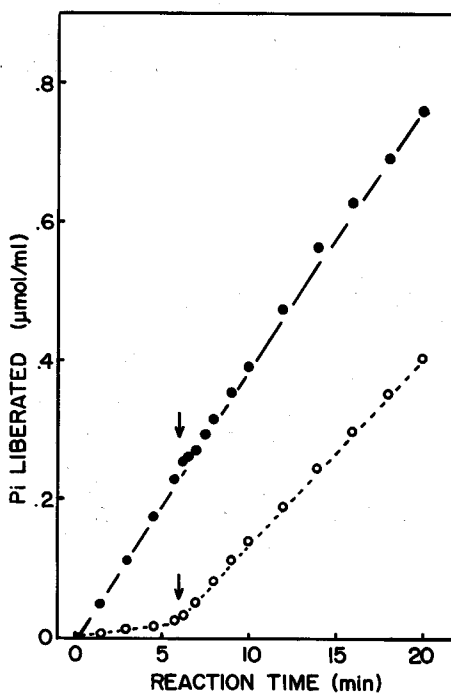


Fig. 45. Effect of urea on the Ca sensitivity of squid myosin Mg-ATPase.

The ATPase activity of squid myosin was assayed in the same medium as in Fig. 38, and at the time indicated by the arrows, 1.1 M urea was added to the reaction mixture without changing other constituents of the medium. The same symbols as in Fig. 44 are also used.

concentration of urea was diluted from 1.2 M to 0.6 M, Pi liberation in the absence of calcium immediately decreased and the myosin recovered Ca sensitivity.

This, therefore, suggested that urea caused some conformational changes in the myosin molecule, which resulted in the loss of Ca sensitivity. However, this change may be small because the lost Ca sensitivity was easily recovered upon the dilution of the urea concentration.

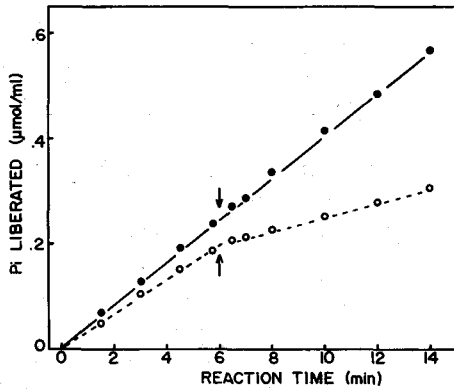


Fig. 46. Recovery of Ca sensitivity of squid myosin Mg-ATPase by dilution of urea.

The ATPase activity of squid myosin was assayed in 30 mM KCl, 20 mM Tris-maleate (pH 6.8), 2 mM MgCl₂, 1 mM ATP, 1.1 M urea and either 0.1 mM CaCl₂ (●) or 0.5 mM EGTA (○). At the time indicated by the arrows, the urea concentration in the reaction mixture was decreased to 0.6 M without changing other constituents of the medium.

2-F-f *Effect of tryptic digestion of squid myosin on Ca sensitivity of its ATPase*

The effect of tryptic digestion of squid myosin on its Ca sensitivity was studied. Squid myosin in 0.5 M KCl, 20 mM Tris-maleate (pH 7.5) was digested by trypsin in a ratio of 1/500 for trypsin/myosin by weight at 10°C. The digestion was stopped by the addition of trypsin inhibitor (5 times the weight of

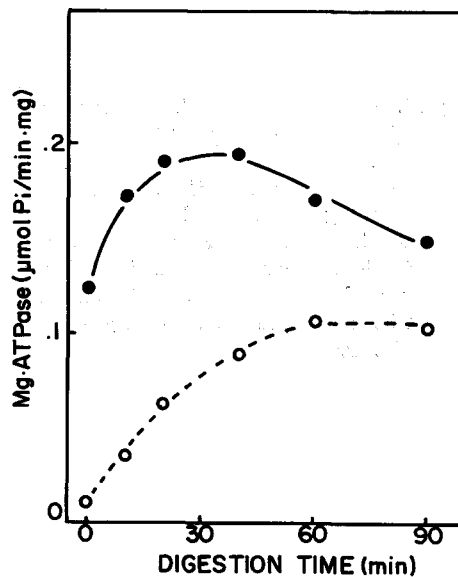


Fig. 47. Change in Mg-ATPase of squid myosin during tryptic digestion.

Squid myosin was digested with trypsin at 10°C in an amount of 1/500 for trypsin/myosin by weight in the medium of 0.5 M KCl, 20 mM Tris-maleate (pH 7.5). The ATPase of squid myosin was assayed in the same medium as in Fig. 38, and the same symbols are used.

trypsin). The change in Mg-ATPase activity is shown in Fig. 47, Mg-ATPase activity of myosin in the absence of calcium gradually increased with increased digestion time and resulted in the loss of Ca sensitivity; Ca sensitivity of squid myosin was lost when the tryptic digestion was continued for 60–90 min.

In addition, the Ca sensitivity of the water-soluble fraction obtained by tryptic digestion was also measured. The digest of squid myosin was dialyzed overnight against 0.05 M KCl, 20 mM Tris-HCl (pH 7.5) and then centrifuged at $100,000 \times g$ for 60 min. The supernatant thus obtained was used for the Mg-ATPase assay. As shown in Table 3, the water-soluble fraction obtained after five minutes digestion was still highly sensitive to calcium. However, when myosin was digested for longer time (30–45 min), the Ca sensitivity of the soluble fraction decreased.

Table 3. Ca sensitivity of water-soluble fractions obtained from tryptic digestion of myosin.

Water-soluble fraction of squid myosin digest was obtained by the following procedures; squid myosin digest obtained as described in Fig. 47 was dialyzed against 0.05 M KCl, 20 mM Tris-HCl (pH 7.5) and centrifuged at $100,000 \times g$ for 60 min. The supernatant thus obtained was used as water-soluble fraction. ATPase was assayed as in Fig. 47, and Ca sensitivity was calculated as described in Fig. 31.

Digestion time (min)	Mg-ATPase ($\mu\text{mol Pi/min/mg}$)		Ca-sensitivity (%)
	+Ca	-Ca	
5	0.189	0.009	95
10	0.201	0.043	79
20	0.211	0.071	66
40	0.214	0.093	57

As shown in Fig. 48, it was also found that the regulatory light chain (squid LC-2) was gradually digested under these conditions. The digestion of the light chain seemed to be proportional to the loss of Ca sensitivity. These results also indicated that the regulatory light chain was essential for the development of Ca sensitivity of myosin alone.

In addition, the effect of intact squid regulatory light chain (squid LC-2) on the Ca sensitivity of the tryptic digest of squid myosin was studied. As shown in Fig. 49, Ca sensitivity of Mg-ATPase of squid myosin digest was unchanged even when an excess amount of squid LC-2 was added. These results strongly suggested that not only the LC-2 molecule itself but also the LC-2 binding site in squid myosin molecule was digested by trypsin.

2-F-g *Ca sensitivity of scallop myosin alone and its loss by EDTA-treatment*

It was important to determine whether or not the Mg-ATPase activity of scallop myosin alone was also sensitive to calcium. As shown in Fig. 50A, Mg-ATPase of scallop myosin, as well as that of squid myosin, was activated by the

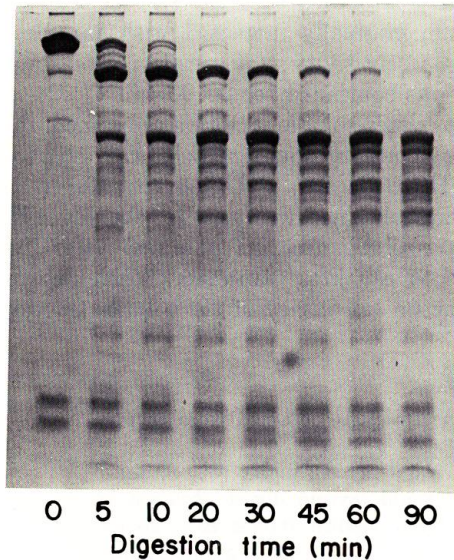
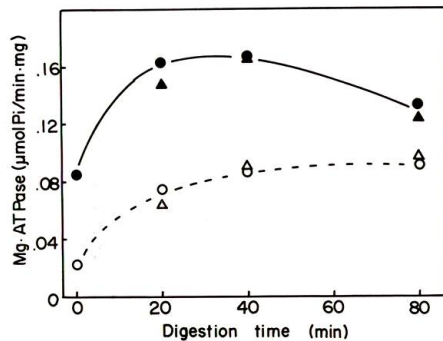


Fig. 48. Change in SDS gel electrophoretic patterns of squid myosin by tryptic digestion.

Squid myosin was digested as described in Fig. 47, and the whole digest of myosin was applied on 10% polyacrylamide gel containing 0.1% SDS.

Fig. 49. Effect of intact squid LC-2 on the Ca sensitivity of Mg-ATPase of squid myosin digest.

Squid myosin was digested by trypsin as described in Fig. 47, and approximately 10% (w/w) of squid LC-2 was then added to myosin digest. The ATPase was assayed as in Fig. 38. (●, ○), squid myosin digest alone; (▲, △), myosin digest with squid LC-2.



same concentrations (1–10 μM) of free calcium as that of acto-scallopy myosin. However, it should be noted that scallop myosin showed a higher ATPase activity than squid myosin in the absence of calcium.

Then, using scallop desensitized myosin, the author studied the role of the regulatory light chain in the Ca sensitivity of myosin alone. Figure 50 shows that the Mg-ATPase activity of desensitized scallop myosin as well as that of acto-desensitized scallop myosin lost Ca sensitivity. The ATPase activity of scallop myosin in the presence of calcium (10–100 μM free calcium) was unaffected by EDTA-treatment, whereas the activity in the absence of calcium (0.1–1 μM free calcium) was greatly increased by the same treatment, resulting in loss of Ca sensitivity. The Ca sensitivity of the ATPase activity of acto-scallop myosin was lost upon EDTA-treatment, but the ATPase activity in the presence of calcium was also reduced by the same treatment (B). Ca sensitivity of myosin ATPase and that of actomyosin ATPase and that of actomyosin ATPase were both recovered

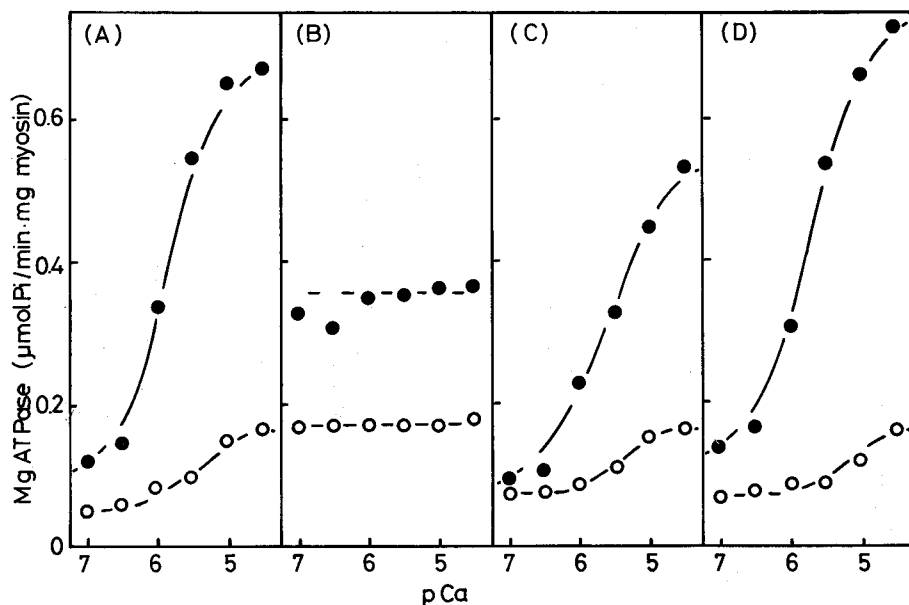


Fig. 50. Calcium requirement for the activation of Mg-ATPase of scallop myosins and of rabbit actomyosins.

ATPase was assayed under the conditions described in Fig. 37, and the same symbols as in Fig. 37 are used. (A) Scallop untreated myosin; (B) scallop desensitized myosin; (C) scallop resensitized myosin with squid LC-2; and (D) scallop resensitized myosin with scallop EDTA-LC.

on the addition of either squid LC-2(C) or scallop EDTA-LC(D). Moreover, the reduced ATPase activity of actomyosin (B) was also recovered on the addition of regulatory light chains.

The effect of the regulatory light chain on the Ca sensitivity of myosin alone, and on that of actomyosin were then studied in detail. Various amounts of regulatory light chain were added to desensitized scallop myosin, and its ATPase activity was measured. As shown in Fig. 51, Mg-ATPase activity of desensitized scallop myosin in the presence of calcium was unaffected by the addition of regulatory light chain, while it was strongly inhibited in the absence of calcium.

With respect to the Mg-ATPase of actomyosin, the ATPase activity of actomyosin in the presence of calcium was elevated upon the addition of regulatory light chain, and the ATPase activity in the absence of calcium was considerably inhibited by the addition of regulatory light chain. The inhibition of the ATPase activity of myosin in the absence of calcium and the elevation of actomyosin ATPase in the presence of calcium both reached a maximum value upon the addition of 7% (w/w) of regulatory light chain to desensitized scallop myosin. This value corresponded to two mol of light chain per mol of desensitized myosin.

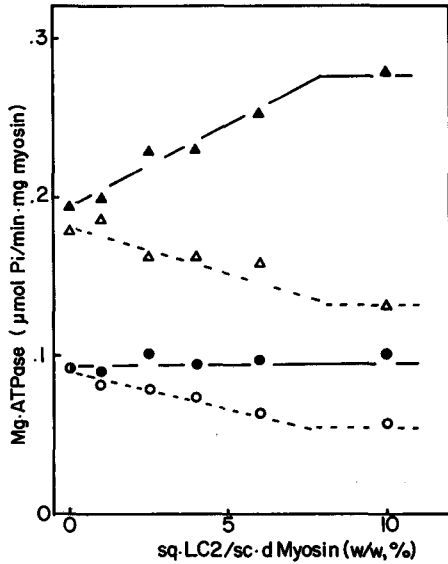
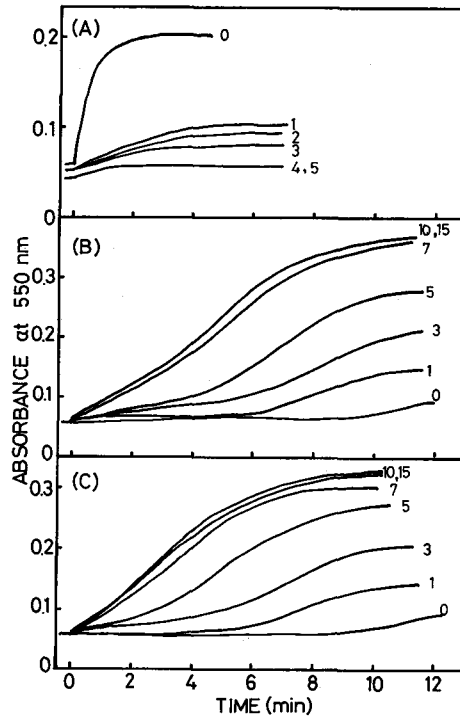


Fig. 51. Effect of squid LC-2 on the Mg-ATPase of desensitized scallop myosin and on that of acto-desensitized myosin.

The ATPase activity was assayed in a medium containing 30 mM KCl, 20 mM Tris-maleate (pH 6.8), 2 mM MgCl₂, 1 mM ATP and either 0.1 mM CaCl₂ (closed) or 0.5 mM EGTA (open.) (●, ○), myosin ATPase; (▲, △), actomyosin ATPase.

Fig. 52. Effects of desensitization and re-sensitization on the superprecipitation of rabbit acto-scallop myosin.

Superprecipitation was measured in a medium containing 30 mM KCl, 20 mM Tris-maleate (pH 6.8), 2 mM MgCl₂, and 0.5 mM ATP. The turbidity (absorbance) change of actomyosin suspension after the addition of ATP was followed at 550 nm. (A) Acto-desensitized scallop myosin; numbers in the figure denote the numbers of repetitions of EDTA-treatment. (B) and (C) Acto-resensitized scallop myosin; numbers in the figure denote the amounts of squid LC-2 (B) and of scallop EDTA-LC added to desensitized scallop myosin (% w/w).



2-F-h *Effects of desensitization and resensitization on superprecipitation ability of acto-scallop myosin*

Similar results were also obtained by measuring the superprecipitation activity instead of ATPase activity. Figure 52A shows that acto-scallop myosin lost its superprecipitation ability as the EDTA-treatment of scallop myosin progressed, even in the presence of calcium. Figure 52(B and C) shows that the lost ability was recovered on the addition of either squid LC-2 or scallop EDTA-LC. The two regulatory light chains were equally effective in effecting the recovery of superprecipitation ability. The recovery was complete when the amount of regulatory light chains added reached about 7% of that of desensitized scallop myosin, which is approximately equivalent to a molar ratio of 1/1 for light chain/heavy chain. This value (7%) was also equal to that required for the recovery of Ca sensitivity in the ATPase activity (Fig. 32).

2-G ATP concentration dependence of ATPase and superprecipitation

2-G-a *ATP concentration dependence of superprecipitation and ATPase of myosin B of squid and scallop*

In the previous section, the Ca sensitivity of myosin alone and that of actomyosin were studied at a fixed ATP concentration of 1 mM. The author then studied this Ca sensitivity at various ATP concentrations.

Figure 53 shows the ATP concentration dependence of the superprecipitation activity of myosin B's from squid and scallop muscles. Superprecipitation occurred in the absence of calcium when the ATP concentration was very low (1–10 μ M). Moreover, superprecipitation of both myosin B's was practically insensitive to

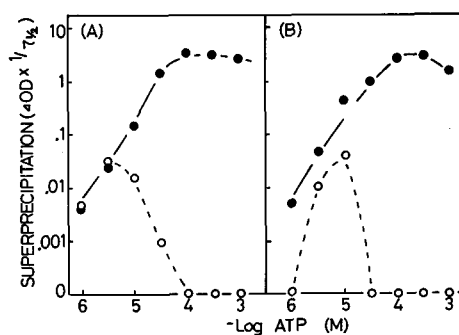


Fig. 53. ATP concentration dependence of the superprecipitation activities of myosin B's from squid and scallop muscle.

Superprecipitation was measured in a medium containing 30 mM KCl, 20 mM Tris-maleate (pH 6.8), 2 mM $MgCl_2$, either 0.1 mM $CaCl_2$ (●), or 0.5 mM EGTA (○) and various concentrations of ATP (1 μ M–1 mM). Pyruvate kinase (0.3 mg/ml) and phosphoenol pyruvate (1 mM) were added to maintain the ATP concentration. Superprecipitation activity was expressed in terms of $\Delta A \times 1/\tau_{1/2}$ where ΔA is the maximal increase in turbidity, and $\tau_{1/2}$ is the time required to reach the half-maximal increase. (A) Squid myosin B. (B) Scallop myosin B.

calcium at 1–10 μM ATP. The superprecipitation activity of both myosin B's, both in the presence and absence of calcium, responded in a biphasic manner to changes in the ATP concentration. The maximal activity was obtained at about 3–10 μM ATP in the absence of calcium and at 100–300 μM ATP in the presence of calcium. Figure 54 shows that the ATPase activities of scallop and squid myosin B's responded essentially in the same manner as their superprecipitation activities (Fig. 53).

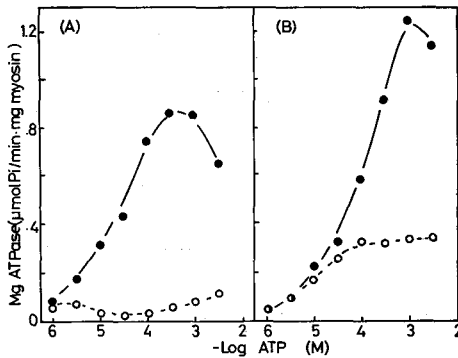


Fig. 54. ATP concentration dependence of Mg-ATPase of myosin B's from squid and scallop muscle.

The ATPase activity of myosin B was assayed under the conditions described in Fig. 53, and the same symbols as in Fig. 53 are used. (A) Squid myosin B, and (B) scallop myosin B.

2-G-b ATP concentration dependence of Mg-ATPase activity of squid and scallop myosin

Figure 55 shows that the Mg-ATPase activity of squid myosin and that of scallop myosin responded in a monophasic manner to changes in the ATP concentration. It was also observed (see also Fig. 37, 50) that the Mg-ATPase activity of scallop myosin was higher than that of squid myosin in the absence of calcium at all ATP concentrations tested, and also that Ca sensitivity at 1 μM ATP was more pronounced with squid myosin than with scallop myosin.

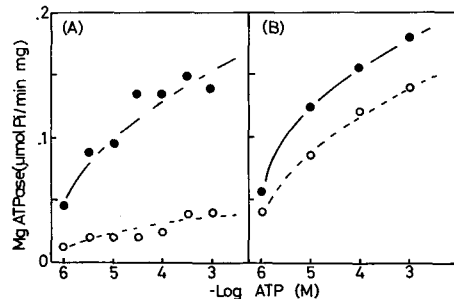


Fig. 55. ATP concentration dependence of Mg-ATPases of myosin from squid and scallop muscle.

The ATPase activity was assayed under the conditions described in Fig. 53, and the same symbols as in Fig. 53 are used. (A) Squid myosin, and (B) scallop myosin.

2-G-c ATP concentration dependence of Mg-ATPase activity of desensitized and resensitized scallop myosin and actomyosin

In order to study the effect of the regulatory light chain on the ATP concentration dependence of myosin alone and on that of actomyosin, the author examined

the ATP concentration dependence of desensitized scallop myosin and that of acto-desensitized scallop myosin as well as those of resensitized scallop myosin and of acto-resensitized myosin. As shown in Fig. 56, the essential features of the ATP concentration dependence of both myosin ATPase and actomyosin ATPase were unaffected by either desensitization or resensitization. For example, as the ATP concentration increased, myosin ATPase increased in a monophasic manner whereas actomyosin ATPase did so in a biphasic manner. It should be pointed out that resensitized myosin with squid LC-2 showed a much lower ATPase activity than did that with scallop EDTA-LC, in the absence of calcium. These differences would arise not from differences in the properties of the heavy chains but from differences in the properties of squid LC-2 and scallop EDTA-LC.

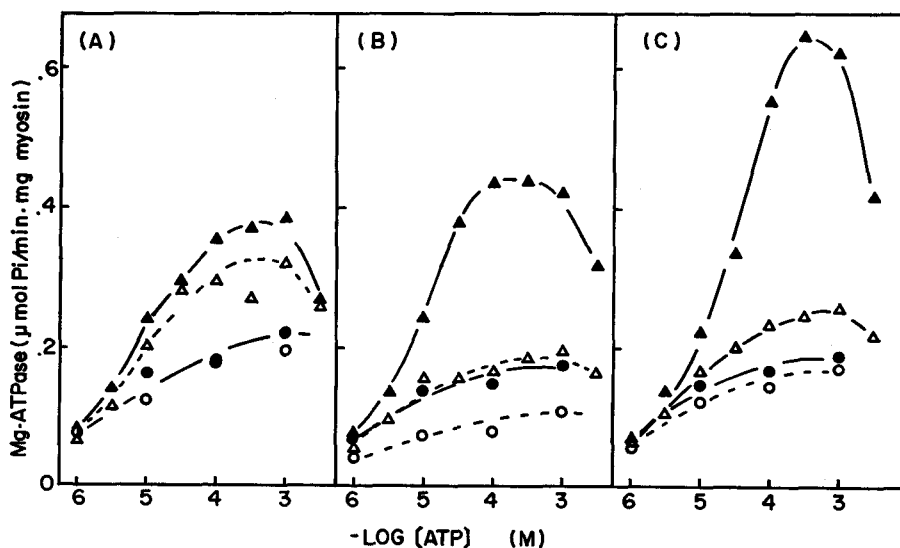


Fig. 56. Effects of desensitization and of resensitization on the ATP concentration dependence of Mg-ATPases of myosin and of actomyosin from scallop muscle.

The ATPase activity was assayed under the conditions described in Fig. 53. (\bullet , \blacktriangle) 0.1 mM CaCl_2 ; (\circ , \triangle) 0.5 mM EGTA; and (\bullet , \circ) myosin alone; (\blacktriangle , \triangle) actomyosin. (A) Desensitized scallop myosin, (B) resensitized myosin with squid LC-2, and (C) resensitized myosin with scallop EDTA-LC.

2-H Tension development of glycerinated muscle fibers of scallop adductor muscle

2-H-a Effect of EDTA-treatment of glycerinated fibers on the Ca sensitivity in tension development

In the previous study on the biochemical role of the regulatory light chain, it was found that a removal of two mol of EDTA-LC was required to desensitize scallop myosin, and that the removal of EDTA-LC remarkably reduced the superprecipitation activity of acto-desensitized myosin.

Next, the role of regulatory light chain was studied by measuring the physiological properties of tension development. The glycerinated muscle fiber from scallop adductor striated muscle was prepared by using the usual method for that from rabbit psoas muscle. The details were described in "Methods and materials".

In Fig. 57A, a typical tension development of scallop muscle fibers is shown. The tension developed only in the contracting solution, and the average tension developed in the presence of calcium was ca. 112 g/cm². As shown in Fig. 57B, when the fibers were treated with EDTA solution for 15 min, as described in "Methods and materials", scallop muscle fibers became capable of developing tension in the absence of calcium. Moreover, it was observed that tension development in the presence of calcium was depressed by the same treatment.

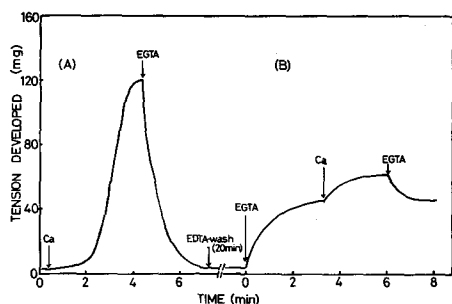
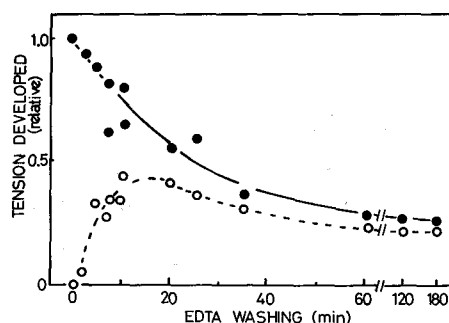


Fig. 58. ATP-induced tension development in glycerinated fibers of scallop adductor muscle in the presence and absence of calcium.

Glycerinated fiber bundles (each approximately 300 μ thick and 5 mm long) were treated with 10 mM EDTA at 20°C for various periods. The extent of tension developed was measured at 20°C in a fiber bath containing 0.1 M KCl, 20 mM imidazole-HCl (pH 6.8), 0.3 mM DTT, 5 mM MgCl₂, 4 mM ATP, and 2 mM EGTA (○) or 0.2 mM CaCl₂ plus 0.1 mM EGTA (●).



In Fig. 58, tension development in the presence of calcium and that in the absence of calcium are both shown as a function of the duration of EDTA-treatment. When the fibers were treated with EDTA solution for 10 min, glycerinated fibers became capable of developing tension in the absence of calcium. The tension developed was nearly half that developed by untreated glycerinated fibers in the presence of calcium. However, it was also observed that tension developed in the presence of calcium was decreased to about three-fourth by the same treatment of

Fig. 57. ATP-induced tension development in glycerinated fibers of scallop adductor muscle.

A typical record of tension development in glycerinated muscle fibers of scallop is shown. Tension development in fibers of scallop was measured in a medium containing 0.1 M KCl, 20 mM imidazole-HCl (pH 6.8), 0.3 mM DTT, 5 mM MgCl₂, 4 mM ATP, and either 2 mM EGTA (EGTA) or 0.2 mM CaCl₂ plus 0.1 mM EGTA (Ca) at 20°. Untreated fibers (A) and EDTA-treated fibers for 20 min (B) were used.

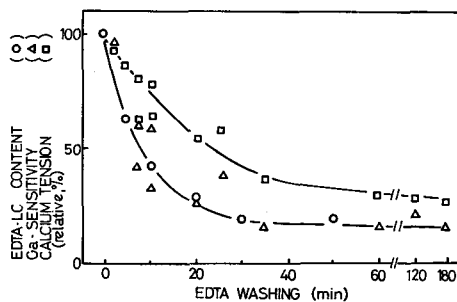
fibers, and that the tension developed continued to decrease to less than one-third that before the EDTA-treatment.

2-H-b *Relationship between EDTA-LC content in fibers and Ca sensitivity of tension development in the presence of calcium*

In the polyacrylamide gel electrophoreses of glycerinated fibers in the presence of either urea or SDS, practically no change was detectable in the contents of heavy chain, actin, tropomyosin, paramyosin and SH-LC during EDTA-treatment. The only change detectable was in the EDTA-LC content. As shown in Fig. 59, the content of EDTA-LC decreased exponentially to about 15% of the initial content, and the half-maximal decrease occurred after about 6 min of EDTA-treatment. Ca sensitivity was estimated from the data shown in Fig. 58, expressed in terms of $(1 - T_E/T_{Ca}) \times 100$, where T_E and T_{Ca} were tension developed in the absence and presence of calcium, respectively. The results indicated that Ca sensitivity of tension development decreased essentially in the same manner as the EDTA-LC content did, the half-maximal decrease occurring after approximately 6 min of the EDTA-treatment. Tension developed in the presence of calcium decreased exponentially, and the half-maximal decrease occurred at 15 min of EDTA-treatment.

Fig. 59. Effect of EDTA-treatment of glycerinated fibers of scallop on the EDTA-LC content, on the Ca sensitivity in tension development, and on the active (calcium) tension.

The EDTA-treatment was conducted at 20°C as described in Fig. 58. The regulatory light chain (EDTA-LC) content (○) was estimated by urea-gel electrophoresis. Ca sensitivity (Δ) and active (calcium) tension (□) were obtained from the data shown in Fig. 58.



2-H-c *Effect of regulatory light chain on Ca sensitivity and on tension development in the presence of calcium*

Moreover, it was found that the ability of developing tension lost in the presence of calcium and the Ca sensitivity in tension development which was lost were both fully recovered. As shown in Fig. 60A and 60B, when the desensitized fibers treated with EDTA for 60 min were soaked in a solution containing either scallop (A) or squid (B) regulatory light chain for recombination, the fibers regained the ability to develop tension in the presence of calcium as much as about 70–90% of that developed by untreated fibers, and they developed no tension in the absence of calcium. Therefore, the recovery of active tension (tension in the presence of calcium) as well as that of Ca sensitivity were very good. In both cases, the time required for the recombination of regulatory light chain to EDTA-treated muscle fibers was about 30 min.

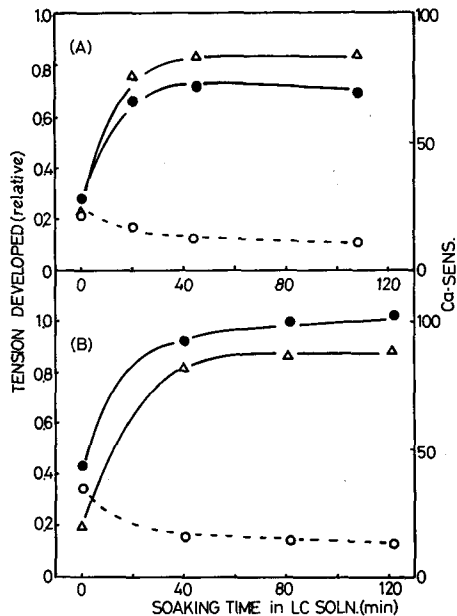


Fig. 60. Resensitization of glycerinated fibers of scallop muscle by regulatory light chains from squid and scallop muscle.

EDTA-treated fibers of scallop for 60 min at 20°C as described in Fig. 58, were soaked in a solution containing 0.1 M KCl, 20 mM imidazole (pH 6.8), 5 mM MgCl₂, 4 mM ATP, 0.3 mM DTT, 2 mM EGTA and squid LC-2 (A) (1.8 mg/ml) or scallop EDTA-LC (B) (1.8 mg/ml) for various lengths of time. The tension developed in the presence (●) and absence (○) of calcium were measured as described in Fig. 57, and Ca sensitivity (Δ) in tension developed are estimated as in Fig. 59.

IV Discussion

SZENT-GYÖRGYI *et al.*⁶⁾ reported an important discovery that muscle contraction in scallop adductor muscle is regulated not by troponin-tropomyosin system but by the myosin molecule. They concluded that all of the molluscan muscles are regulated only by a myosin-linked Ca regulatory system. Up to the present time, few studies have reported on the Ca regulation of molluscan muscle beyond those of SZENT-GYÖRGYI and his group.

The author has studied at same length the mechanism of Ca regulation in molluscan muscle, especially in squid and scallop muscle, which are both categorized as members of a group containing only myosin-linked regulation by LEHMAN and SZENT-GYÖRGYI.⁶⁾ However, in applying the competitive test⁶⁾²⁴⁾ for squid myosin B, one finds evidence for both myosin-linked and actin-linked Ca regulations in squid myosin B, a conclusion which conflicts with the report of LEHMAN *et al.*²⁴⁾ Since two Ca regulatory systems were assumed in squid muscle, the author tried to ascertain whether squid muscle truly contains two regulation systems or not.

Initially, actin-linked Ca regulation in squid muscle was studied. Ca sensitivity in the Mg-ATPase of actomyosin constituted from squid actin and rabbit myosin was measured. The author found that actomyosin from purified squid actin is not Ca sensitive, while the actomyosin formed using crude squid actin is sensitive to calcium. These results suggested that squid crude actin contains protein components which confer Ca sensitivity on actomyosin. Accordingly, an attempt was made to isolate native tropomyosin and biologically active native tropomyosin and troponin were both obtained from squid muscle. Squid native tropomyosin, thus obtained, confers Ca sensitivity on rabbit actomyosin just

as rabbit native tropomyosin does. This suggests the functional identity of squid native tropomyosin with rabbit native tropomyosin, though squid native tropomyosin is less effective in this regard than rabbit native tropomyosin, *i.e.* the inhibition of actomyosin Mg-ATPase activity by squid native tropomyosin in the absence of calcium is relatively small. However, squid native tropomyosin, as well as rabbit, was found to be a complex of troponin and tropomyosin, which were both successfully separated and isolated. Squid troponin also contains three subunits (52,000, 28,000 and 24,000 daltons) (Fig. 8). The 52,000 daltons component has been widely observed in troponin preparations from various arthropod (Lobster, crayfish and *Limulus*)²⁷⁾³¹⁾⁴¹⁾⁴²⁾. Moreover, arthropods occupy a position just above mollusca on the phylogenetic tree. These facts may be relevant to the speculations concerning the evolution of the regulatory protein system as LEHMAN *et al.* have proposed⁶⁾. The squid troponin subunit of 52,000 daltons was found to be capable of binding tropomyosin. Since the 52,000 dalton component in lobster troponin corresponds to the TN-T in rabbit troponin, which is its tropomyosin binding component, this 52,000 dalton component in squid troponin also corresponds to the TN-T of mammalian muscle. It was found that the squid troponin is present in the form of complex of its three components under physiological conditions, and that the complex is easily dissociated by low concentrations of urea (0.1–0.2 M). These results suggest that the 3 subunits of squid troponin are weakly bound in their complex. It was also found that squid native tropomyosin as well as squid troponin can bind only a small amount of calcium (0.2 mol of Ca/ 1×10^5 g protein). These two prospects may be included in the reason for the characteristic properties of squid native tropomyosin in conferring Ca sensitivity.

Myosin-linked Ca regulation, especially in scallop adductor muscle, has been studied by SZENT-GYORGYI⁶⁾ and his associates. The author's results essentially confirmed their results in that actomyosin reconstituted from squid myosin and rabbit purified actin is sensitive to calcium in ATPase and in superprecipitation. These results clearly show that squid muscle contains a myosin-linked regulation system besides an actin-linked regulation system as described above, and the two regulatory system are found to be functional, if tested separately.

There are a few reports suggesting the presence of both myosin-linked and actin-linked regulation in other systems, *e.g.*, in insect flight and leg muscles⁶⁾, in earthworm body wall⁶⁾, and in *Physarum polycephalum*⁴³⁾. However, there have been no reports presenting direct evidence, *i.e.* isolation and characterization of both systems. Therefore, the present report on two Ca regulation systems in squid mantle muscle may provide this direct evidence for the first time.

Naturally, the question may arise as to which of the two regulatory systems predominates in squid muscle. Fortunately, these two systems are differentiated by their Ca and Sr concentration requirements for the activation of their respective Mg-ATPase activity, *i.e.* actin-linked regulation requires lower Ca and Sr concentrations for the activation of its Mg-ATPase than the myosin-linked regulation system does. KENDRICK-JONES *et al.*²⁾ reported that scallop myosin B Mg-ATPase (myosin-linked) is fully activated only at higher Ca concentration than is required for rabbit skeletal myosin B Mg-ATPase. The Ca requirement for

activation of squid myosin B, which indeed contains both systems, was found to be identical with that for the activation of the myosin-linked system. It should be noted that the Sr requirement of squid crude acto-rabbit myosin (actin-linked) is identical with that of rabbit myosin B. Therefore, it seems that the myosin-linked system rather than the actin-linked system predominates. This is further supported by the fact that an addition of a large amount of native tropomyosin from squid or rabbit does not shift the Ca requirement of squid myosin B from that of the myosin-linked system to that of the actin-linked system.

A further study of the myosin-linked Ca regulation in squid muscle was made. In scallop muscle, it is well known that the regulatory subunit of myosin is one type of light chain (EDTA-LC)⁴⁾, which is able to be isolated by the EDTA-treatment of myosin. The author tried to isolate the regulatory light chain from squid myosin. As already described, none of the light chains are released from myosin by treatment with EDTA, but they are released from myosin by treatment with urea. The mixture of light chains thus released are successfully separated by using DEAE-Sephadex column chromatography. One of the light chains (LC-2) was considered to be the calcium binding subunit, since calcium induces a spectral change in LC-2 and also induces a mobility change in LC-2 in disc gel electrophoresis. The calcium binding ability of squid LC-2 was measured by the equilibrium dialysis method, and isolated LC-2 was found to bind a small amount of calcium (0.4–0.5 mol of Ca/mol of LC-2), this value is somewhat smaller than that obtained with intact squid myosin (2 mol of Ca/mol of myosin, or 1 mol of Ca/mol of LC-2). These results suggest that when LC-2 is released from myosin, its conformation, probably at the calcium binding site, may be changed and thus cause a decrease in its calcium binding ability. It should be pointed out that squid LC-2 has a better calcium binding ability than scallop EDTA-LC (0.15 mol of Ca/mol of EDTA-LC).

In order to test the biological function of squid regulatory light chain (LC-2), scallop myosin was used, because squid myosin could not be desensitized by EDTA-treatment or by any other procedure. In the process of desensitization of scallop myosin, the author found results which conflict with those of SZENT-GYÖRGYI *et al.*⁶⁾. For instance, they reported that among four mol of light chains (2 mol of SH-LC, 1 mol of EDTA-LC and 1 mol of DTNB-LC) in scallop myosin, the removal of 1 mol of EDTA-LC is enough to desensitize scallop myosin. Based on these results, they have proposed a functional cooperativity between the two heads of myosin or two mol of light chains in the molecular mechanism of Ca regulation. On the other hand, the present author found that the removal of 2 mol of EDTA-LC is required to desensitize scallop myosin, *i.e.* Ca sensitivity is still maintained even if 1 mol of EDTA-LC is removed from myosin. Moreover, it was found that two mol of EDTA-LC are easily removed by EDTA-treatment, and that DTNB-LC cannot be distinguished from EDTA-LC by this technique. These results suggest the absence of any functional cooperativity of the light chains in the Ca regulation mechanism.

Desensitized scallop myosin was used for the functional test of the regulatory light chain of squid, by studying the Mg-ATPase activity of reconstituted actomyosin. Squid LC-2 is capable of binding desensitized scallop myosin and confers on it Ca sensitivity, the binding of LC-2 and the recovery of Ca

sensitivity are both maximized at 2 mol of squid LC-2 added per mol of myosin. Therefore, squid LC-2 is equivalent to scallop EDTA-LC and is not distinguishable from EDTA-LC in its function. Furthermore, the squid LC-2 bound to desensitized myosin is easily removed by EDTA-treatment. Therefore, it seems likely that the difficulty experienced in removing the light chain from squid myosin by EDTA-treatment is not caused by the properties of the myosin regulatory light chain but by those of the myosin heavy chain.

Recently, ASADA *et al.*³⁹⁾ reported that the Mg-ATPase of clam foot muscle myosin alone is sensitive to calcium as well as actomyosin. The author studied whether this property of clam foot myosin was restricted to clam or was a general property of all molluscan myosin. As a result, squid myosin Mg-ATPase was found to be clearly activated by low concentrations of calcium ions between 1 μM and 10 μM . Moreover, the Ca requirement for activation of myosin Mg-ATPase was identical with that required for the activation of actomyosin Mg-ATPase. One, therefore, concludes that the Mg-ATPase of squid myosin alone is sensitive to calcium. Changes in temperature and pH used for the Mg-ATPase assay had no effect on the Ca sensitivity of myosin alone. However, KCl and urea did affect the Mg-ATPase activity and Ca sensitivity of myosin alone. The Ca sensitivity of myosin Mg-ATPase is lost at KCl concentration higher than 0.4 M. On the other hand, the actin activation of Mg-ATPase of myosin in the presence of calcium is lost to 0.2 M KCl. Therefore, one can distinguish the effect of KCl on the Ca sensitivity of myosin Mg-ATPase and its actin-activation.

In the presence of 1-1.2 M urea, myosin Mg-ATPase is insensitive to calcium, but actin-activation still occurs. At the same time, it was found that actin-activation of myosin Mg-ATPase in the absence of calcium increased, resulting in the loss of the Ca sensitivity of its actin-activation. These results suggest that Ca sensitivity of myosin alone may always be coupled with actin-activation. It appears, therefore, that a primary role of the regulatory light chain is to regulate (inhibit) myosin Mg-ATPase, and that if this inhibition of myosin Mg-ATPase is removed, binding of myosin to actin occurs.

Scallop myosin Mg-ATPase is sensitive to calcium as is squid myosin Mg-ATPase. Ca sensitivity of its Mg-ATPase is lost by the removal of all EDTA-LC from scallop myosin. Moreover, Ca sensitivity of actomyosin measured by Mg-ATPase and by superprecipitation are both lost by the removal of EDTA-LC. It was also found that actin-activation of desensitized scallop myosin Mg-ATPase and superprecipitation of acto-desensitized scallop myosin are remarkably reduced even in the presence of calcium and are both recovered on the addition of either squid or scallop regulatory light chain. These observations suggest that another function of the regulatory light chain is to maintain regular binding of myosin and actin in the presence of calcium. This is further confirmed by measuring the change in the tension development of glycerinated muscle fibers of scallop during EDTA-treatment. Ca sensitivity in the tension development of glycerinated fibers is lost by the removal of their EDTA-LC, while the tension development in the presence of calcium decreased with decreasing amounts of EDTA-LC. Moreover, Ca sensitivity and tension development in the presence of calcium are both recovered upon the addition of either squid or scallop regulatory light chain.

From these results, the author speculates that when light chain is removed from myosin, Ca sensitivity of myosin as well as actomyosin are concomitantly lost, and that the removal of light chain may induce a conformational change in the myosin molecule, and presumably also in thick-filament formation. This change may lead to a decrease in the tension development and in the actin-activation of myosin ATPase.

V SUMMARY

Squid mantle myosin B was Ca sensitive, and its Ca sensitivity was unaffected by the addition of large amounts of rabbit skeletal myosin or rabbit skeletal F-actin. This suggests that both myosin-linked and actin-linked Ca regulatory systems are present in squid mantle muscle.

1 Actin-linked regulatory system

Squid mantle actin was prepared by the method of SPUDICH and WATT. Hybrid actomyosin, reconstituted using the purified squid actin and skeletal myosin, showed no Ca sensitivity in the Mg-ATPase assay, whereas that reconstituted using crude squid actin showed a marked Ca sensitivity. The crude squid actin contained four protein components which were capable of associating with F-actin in 0.1 M KCl, 1 mM MgCl₂ and 20 mM Tris-maleate (pH 7.5).

Native tropomyosin was prepared from squid mantle muscle, and conferred Ca sensitivity on skeletal actomyosin as well as on hybrid actomyosin reconstituted from squid actin and skeletal myosin, but it is less effective than skeletal native tropomyosin in conferring this Ca sensitivity.

Squid native tropomyosin was separated into troponin and tropomyosin fractions by placing it in 0.4 M LiCl at pH 4.7. The troponin fraction was further purified by DEAE-cellulose chromatography. Squid troponin thus obtained was different in mobility in SDS gel electrophoresis from rabbit skeletal troponin; three bands of squid troponin corresponded to molecular weights of 52,000, 28,000 and 24,000 daltons. It could confer Ca sensitivity in the presence of tropomyosin on skeletal actomyosin as well as on hybrid actomyosin reconstituted from squid actin and skeletal myosin. It was also found that the troponin-tropomyosin complex using squid troponin was less effective in this regard than that formed using skeletal troponin.

Squid troponin could not bind F-actin alone, whereas it could bind F-actin in the presence of tropomyosin from squid or skeletal muscle.

2 Myosin-linked regulatory system

Squid myosin was prepared from the mantle muscle, which showed a heavy chain component and two light chain components in a SDS gel electrophoretic pattern: the molecular weights of the latter two were 17,000 and 15,000 daltons. Actomyosin reconstituted from squid myosin and skeletal (or squid) actin showed Ca sensitivity in superprecipitation and in Mg-ATPase assay. EDTA-treatment had no effect on the Ca sensitivity of squid myosin.

Squid myosin B and two hybrid actomyosin were compared as regards their Ca and Sr requirements in Mg-ATPase activities. This result indicated that myosin-linked regulatory system predominates in squid myosin B. Squid myosin B required higher Ca or Sr concentration for Mg-ATPase activity: half maximal activation of Mg-ATPase was obtained at $0.8 \mu\text{M}$ calcium ions and at $28 \mu\text{M}$ strontium ions with skeletal myosin B, and at $2.5 \mu\text{M}$ calcium ions and at $140 \mu\text{M}$ strontium ions in the case of squid myosin B.

The author was able to separate two types of light chain by a five steps procedure, yielding LC-1 (17,000 daltons) and LC-2 (15,000 daltons). It was found that squid mantle LC-1 and LC-2 functions exactly like SH-LC and EDTA-LC respectively of scallop adductor myosin.

Calcium or strontium ions were found to induce changes in the UV absorption spectrum of scallop adductor EDTA-LC, although the apparent binding constants estimated from the difference spectrum were too low to account for the Ca sensitivity of scallop actomyosin Mg-ATPase. The divalent cations also induced changes in the UV absorption spectrum of squid LC-2, and the apparent binding constants estimated from the difference spectrum were sufficiently high ($1.5 \times 10^5/\text{M}$ for Ca binding, and $1.6 \times 10^3/\text{M}$ for Sr binding) to account for the Ca and Sr sensitivities of squid myosin B ATPase.

In contrast with the results of KENDRICK-JONES *et al.*, the author showed that the removal of two moles of EDTA-LC from scallop myosin was required to desensitize actomyosin Mg-ATPase. Desensitized scallop myosin was capable of regaining its full content of EDTA-LC (two moles) as well as its full recovery of Ca sensitivity of actomyosin Mg-ATPase. It was also found that as regards its ability to combine with, and to confer Ca sensitivity on the desensitized scallop myosin, squid LC-2 could effectively replace scallop EDTA-LC.

Calcium binding to the regulatory light chain was studied by an equilibrium dialysis method. It was found that the calcium binding ability of regulatory light chain was decreased from 1 mol of Ca/mol of bound light chain to 0.15–0.4 mol of Ca/mol of isolated light chain and was reduced upon the addition of magnesium ion. Moreover, the calcium binding ability of regulatory light chain fully recovered upon recombination with the myosin molecule.

The Mg-ATPase activity of squid myosin alone was sensitive to calcium. Changes in temperature (5°C – 30°C) and pH (6.0–9.5) for the Mg-ATPase assay left unaffected the Ca sensitivity of squid myosin. The Ca sensitivity of myosin alone was not observed under conditions where the KCl concentration was higher than 0.4 M or when 1.2 M urea was present in the reaction medium. Moreover, the actin-activation of myosin Mg-ATPase in the presence of calcium was lost at a KCl concentration higher than 0.2 M. Therefore, the effects of KCl on the Ca sensitivity of myosin alone and on the actin-activation were able to be distinguished. Tryptic digestion of squid myosin also decreased the Ca sensitivity upon the digestion of LC-2.

Scallop myosin was also sensitive to calcium, and its Ca sensitivity was lost by the removal of EDTA-LC. On the addition of squid LC-2 or scallop EDTA-LC, the Ca sensitivity of desensitized scallop myosin was recovered. Furthermore, removal of EDTA-LC from scallop myosin resulted in a reduction the superprecip-

tation and Mg-ATPase activities of actomyosin, but they were recovered on the addition of regulatory light chains from squid or scallop.

Removal of all of the regulatory light chain (EDTA-LC) of myosin in glycerinated fibers of scallop adductor striated muscle resulted in an immediate loss of Ca sensitivity in tension development and a concomitant decrease in tension development in the presence of calcium. These two properties of glycerinated muscle fibers were recovered by soaking the fibers in a solution containing regulatory light chain from squid or scallop.

The results described above show that squid mantle muscle contains not only a myosin-linked but also an actin-linked regulation system, although the myosin-linked rather than actin-linked regulation is operates in squid myosin B. A further conclusion is that a primary role of the regulatory ight chain of squid or scallop myosin is to inhibit myosin Mg-ATPase, leading to the inhibition of actin-myosin binding in the absence of calcium. Moreover, the regulatory light chain was found to be essential for the regular binding of myosin to actin in the presence of calcium, which is considered to be a basic reaction in the muscle contraction mechanism.

VI Acknowledgements

I wish to express my sincere thanks to Professor S. Watanabe and to Dr. K. Arai for their helpfull advice and encouragement throughout the period of this investigation.

My special thanks go to Dr. Joseph A Duke, University of California, San Francisco, for his review of the manuscript.

I am gratefull also to Professor M. Akiba, T. Yasui and to T. Shibata for their critical review of the manuscript.

To Mrs. K. Ishida and B. Yoshitomi, I wish to express gratitude for their technical assistance.

Finally my deepest thanks go to my wife Yoshiko for her enduring encouragement.

VII References

- 1) EBASHI, S. (1963). Third component participating in the superprecipitation of "natural actomyosin". *Nature*. **200**, 1010.
- 2) KENDRICK-JONES, J., LEHMAN, W. and SZENT-GYÖRGYI, A.G. (1970). Regulation in molluscan muscles. *J. Mol. Biol.* **54**, 313-326.
- 3) EBASHI, S. and EBASHI, F. (1963) A new protein component participating in the superprecipitation of myosin B. *J. Biochem.* **55**, 604-613
- 4) SZENT-GYÖRGYI, A.G., SZENT-KIRALYI, E.M. and KENDRICK-JONES, J. (1973) The light chains of scallop myosin as regulatory subunits. *J. Mol. Biol.* **74**, 179-203.
- 5) KENDRICK-JONES, J. (1974) Role of myosin light chains in calcium regulation. *Nature*. **249**, 631-634.
- 6) LEHMAN, W. and SZENT-GYÖRGYI, A.G. (1975) Regulation of muscle contraction. Distribution of actin control and myosin control in the animal kingdom. *J. Gen. Physiol.* **66**, 1-30.
- 7) EBASHI, S. (1975) A simple method of preparing actin-free myosin from smooth muscles. *J. Biochem.* **79**, 229-231

- 8) SPUDICH, J.A. and WATT, S. (1971) The regulation of rabbit skeletal muscle contraction. *J. Biol. Chem.* **246**, 4866-4871.
- 9) EBASHI, S., KODAMA, A. and EBASHI, F. (1968) Troponin. Preparation and physiological function. *J. Biochem.* **64**, 465-477.
- 10) KONNO, K. (1978) Two calcium regulatory systems in squid muscle. *Ibid.* **83**, 1431-1440.
- 11) HOLT, J.C. and LOWEY, S. (1975) An immunological approach to the role of the low molecular weight subunits in myosin. *Biochemistry.* **14**, 4500-4609.
- 12) PERRIE, W.T., SMILLIE, L.B. and PERRY S.V. (1973) A phosphorylated light-chain component of myosin from skeletal muscle. *Biochem. J.* **135**, 151-164.
- 13) CONNELL, J.J. (1954) Preparation and properties of cod myosin. *Ibid.* **58**, 360-367.
- 14) SZENT-GYÖRGYI, A. (1949) Free-energy relations and contraction of actomyosin. *Biol. Bull.* **96**, 140-161.
- 15) FISKE, C.H. and SUBBAROW, Y. (1925) The colorimetric determination of phosphorus. *J. Biol. Chem.* **66**, 375-400.
- 16) Reynard, A.M., HAAS, L.F. JACOBSEN, D.D. and BOYER, P.D. (1961) The correlation of reaction kinetics and substrate binding with the mechanism of pyruvate kinase. *Ibid.* **236**, 2277-2283.
- 17) OGAWA, Y. (1968) Apparent binding constant of GEDTA to calcium at neutral pH. *J. Biochem.* **64**, 255-260.
- 18) WEBER, K. and OSBORN, M. (1969) The reliability of molecular weight determinations by dodecyl sulfate polyacrylamide gel electrophoresis. *J. Biol. Chem.* **244**, 4406-4412.
- 19) DAVIS, B.J. (1964) Disc electrophoresis-II. Method and application to human serum proteins. *Ann. N.Y. Acad. Sci.* **121**, 404-427.
- 20) POTTER, J.D. and GERGELY, J. (1974) Troponin, tropomyosin, and actin interaction in the Ca reulation of muscle contraction. *Biochemistry.* **13**, 2697-2703.
- 21) KUWAYAMA, H. and YAGI, K. (1977) Separation of low molecular weight components of cardiac myosin subfragment-1, and Ca binding to one of the components. *J. Biochem.* **82**, 25-33.
- 22) GORNALL, A.G., BARDWILL, C.T. and DAVID, M.M. (1949) Determination of serum protein by means of the biuret reaction. *J. Biol. Chem.* **177**, 751-766.
- 23) SIMMONS, R.M. and SZENT-GYÖRGYI, A.G. (1978) Reversible loss of calcium control of tension in scallop striated muscle associated with the removal of regulatory light chain. *Nature.* **273**, 319-320.
- 24) LEHMAN, W., KENDRICK-JONES, J. and SZENT-GYÖRGYI, A.G. (1972) Myosin-linked regulatory systems: Comparative studies. *Cold Spring Harbor Symp. Quant. Biol.* **37**, 319-330.
- 25) TSUCHIYA, T., KANEKO, T. and MATSUMOTO, J.J. (1978) Calcium sensitivity of mantle muscle of squid. *J. Biochem.* **83**, 1191-1193.
- 26) GOLDBERG, A. and LEHMAN, W. (1978) Troponin-like proteins from muscles of the scallop. *Biochem. J.* **171**, 413-418.
- 27) KONNO, K., ARAI, K. and WATANABE, S. (1977) Regulatory proteins from dorsal muscle of the carp. *J. Biochem.* **82**, 931-938.
- 28) EBASHI, S., WAKABAYASHI, T. and EBASHI, F. (1971) Troponin and its components. *Ibid.* **69**, 441-445.
- 29) GREASER, M.L. and GERGELY, J. (1971) Purification and properties of the components from troponin. *J. Biol. Chem.* **246**, 4226-4234.
- 30) WILKINSON, J.M., PERRY, S.V., COLE, H.A. and TRAYER, I.P. (1972) The regulatory proteins of the myofibril. *Biochem. J.* **127**, 215-221.
- 31) REGENSTEIN, J.M. and SZENT-GYÖRGYI, A.G. (1975) Regulatory proteins of lobster striated muscle. *Biochemistry.* **14**, 917-926.
- 32) KENDRICK-JONES, J., SZENT-KIRALYI, E.M. and SZENT-GYÖRGYI, A.G. (1976) Regulatory light chains in myosins. *J. Mol. Biol.* **104**, 747-775.

- 33) TSUCHIYA, T., YAMADA, N. and MATSUMOTO, J.J. (1978) Extraction and purification of squid myosin. *Bull. Japan Soc. Sci. Fish.* **44**, 175-179.
- 34) TSUCHIYA, T., SUZUKI, H. and MATSUMOTO, J.J. (1977) Physicochemical and biochemical properties of squid actin. *Ibid.* **43**, 877-884.
- 35) IKEBE, M., ONISHI, H. and WATANABE, S. (1977) Phosphorylation and dephosphorylation of light chain of the chicken gizzard myosin molecule. *J. Biochem.* **82**, 299-302.
- 36) GAZITH, J., HIMMELFARB, S. and HARRINGTON, W.F. (1970) Studies on the subunit structure of myosin. *J. Biol. Chem.* **245**, 15-22.
- 37) HEAD, J.F., WEEKS, R.A. and PERRY, S.V. (1977) Affinity-chromatographic isolation and some properties of troponin-C from different muscle types. *Biochem. J.* **161**, 465-471.
- 38) YAZAWA, M. and YAGI, K. (1977) A calcium-binding subunit of myosin light chain kinase. *J. Biochem.* **82**, 287-289.
- 39) ASADA, T., ASHIBA, G. and WATANABE, S. (1979) Calcium sensitivity of foot muscle myosin from clam. *Ibid.* **85**, 1543-1546.
- 40) KIMURA, I., ARAI, K., TAKAHASHI, K. and WATANABE, S. (1980) Earliest event in the heat denaturation of myosin. *Ibid.* **88**, 1703-1713.
- 41) BENZONANA, G., KOHLER, L. and STEIN, E.A. (1974) Regulatory proteins of crayfish tail muscle. *Biochem. Biophys. Acta.* **368**, 247-258.
- 42) LEHMAN W., REGENSTEIN, J.M. and RANSON, A.L. (1976) The stoichiometry of the components of arthropod thin-filament. *Ibid.* **434**, 215-228.
- 43) NACHIMIAS, V.T. and ASCH, A. (1976) A calcium sensitive preparation from *Physarum polycephalum*. *Biochemistry.* **15**, 4273-4278.