Biochem. J. (1980) **187**, 447–456
Printed in Great Britain

The Stoichiometry and Location of Troponin I- and Troponin C-Like Proteins in the Myofibril of the Bay Scallop, *Aequipecten irradians*

William LEHMAN, James F. HEAD and Philip W. GRANT
Department of Physiology, Boston University School of Medicine, Boston, MA 02118, U.S.A.

(*Received 22 October 1979*)

Localization and quantification studies were carried out on bay-scallop (*Aequipecten irradians*) striated-muscle troponin C- and troponin I-like proteins. Indirect immunofluorescence microscopy of scallop myofibrils stained with either rabbit anti-(scallop troponin I) or anti-(scallop troponin C) antibodies shows staining of all I-bands observed. The results of quantification studies using sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of untreated scallop myofibrils, washed scallop myofibrils, and isolated scallop thin filaments indicate an actin/tropomyosin/troponin-C molar ratio of 7:1:1. The molar ratio for troponin I could not be determined in untreated myofibrils because of interfering bands; in washed myofibrils a value of 0.6 mol of troponin I/mol of tropomyosin was found. Purified scallop troponin C binds Ca^{2+} and interacts with scallop troponin I to relieve troponin I-induced inhibition of actomyosin ATPase. Although scallop troponin C is an acidic protein, it appears to be less acidic than troponin C from higher organisms. A calmodulin-like protein has been isolated from scallop striated muscle that activates bovine brain phosphodiesterase to the same extent as does brain calmodulin. Its amino acid composition and its electrophoretic mobility on alkaline 6 M-urea/polyacrylamide gels differs from that of scallop troponin C, and it appears not to be associated with thin filaments.

The regulation of contraction of molluscan muscle by Ca^{2+} was previously believed to be solely thick-filament-linked, a function of the myosin molecule. However, the discovery of troponin-like proteins in the striated muscles of the bay scallop (*Aequipecten irradians*) (Lehman & Szent-Györgyi, 1975; Goldberg & Lehman, 1978) and mantle muscle of the squid *Ommastrephes sloani pacificus* (Konno, 1978; Tsuchiya *et al.*, 1978) suggests that a thin-filament-linked regulatory system may be also present, and, in common with many other invertebrates, a dual-linked regulatory system may exist in these molluscs.

Molluscan troponin, like vertebrate troponin, appears to consist of a number of subunits. In vertebrate skeletal muscle, where the mechanism of troponin action has been well characterized, each troponin complex, consisting of three subunits, acts in conjunction with a second thin-filament component, tropomyosin, to control actomyosin ATPase. Reconstitution studies *in vitro* using troponin from the bay scallop shows that this protein is also able to regulate vertebrate actomyosin ATPase. In the vertebrate system, deficiencies in either troponin or tropomyosin, or both, diminish the calcium-dependency of the actomyosin interaction. Whereas comparable amounts of tropomyosin are present in both vertebrate and molluscan muscles, previous estimates have suggested that troponin may be present in molluscan muscle in decreased amounts (Lehman & Szent-Györgyi, 1975; Szent-Györgyi, 1976). If this were correct, then either the troponin in molluscs would be non-functional or functioning by a mechanism distinct from the vertebrate model. The aim of the present study was therefore to determine if troponin in molluscan muscle is present in amounts comparable with troponin in vertebrate muscle and whether its localization and properties would enable it to function in the same manner as the vertebrate troponin.

Abbreviations used: Mg-ATPase, Mg^{2+}-activated actomyosin adenosine triphosphatase; SDS, sodium dodecyl sulphate; IgG, immunoglobulin G.

Materials and Methods

Preparation of scallop proteins

The striated adductor muscles of live bay scallops (*Aequipecten irradians*) were dissected free of smooth muscle, and used in the preparations described below. These preparations were carried out between 0 and 4°C.

0306-3275/80/050447-10$01.50/1

Myofibrils. So-called 'unwashed myofibrils' were prepared by homogenizing three scallop muscles for 6–10s in 60ml of 0.3M-sucrose/0.1M-NaCl/1mM-$MgCl_2$/1mM-EGTA/1mM-NaN_3/10mM-sodium phosphate buffer, pH7.0, with a Sorvall Omni-Mixer. 'Washed myofibrils' were prepared as described previously (Lehman, 1977).

Native and synthetic thin filaments. Native thin filaments were isolated by differential centrifugation as described by Goldberg & Lehman (1978). Synthetic thin filaments were prepared by allowing scallop troponin–tropomyosin to form a complex with rabbit actin (Goldberg & Lehman, 1978).

Actin. Purified actin was extracted from native thin filaments by selectively removing troponin–tropomyosin at high ionic strength (Spudich & Watt, 1971; Lehman *et al.*, 1972).

Tropomyosin. Tropomyosin was prepared as described by Lehman *et al.* (1976).

Troponin C. Troponin C was prepared from native thin filaments by first dissolving the filaments in 6M-urea/0.03% 2-mercaptoethanol/10mM-sodium acetate buffer, pH5.0. This solution was then applied to a 10ml column of SP(sulphopropyl)-Sephadex C-50 equilibrated with the above solution. Troponin C, tropomyosin and actin are not bound and were eluted with three times the void volume. The urea in the eluate was removed by dialysis against 15mM-2-mercaptoethanol and the sample freeze-dried and redissolved in 1ml of 15mM-2-mercaptoethanol/100mM-NaCl/1mM-EDTA/20mM-Tris buffer, pH7.8. Troponin C was then isolated by chromatography on a column (1.5cm × 90cm) of Sephadex G-100 equilibrated with the above solution, and was the last protein eluted. The troponin C was dialysed against 5mM-NaCl, freeze-dried, redissolved in 1–2ml of water and redialysed against 50mM-NaCl. This procedure yields approx. 200–400μg of troponin C from eight to ten muscles.

Troponin I. Troponin I was prepared as described by Goldberg & Lehman (1978).

Calmodulin. Washed myofibrils from five to eight muscles were sedimented and homogenized in 80ml of 100mM-NaCl/1mM-EDTA/6M-urea/10mM-sodium phosphate buffer, pH7.0. Particulate material was removed by centrifugation at 40000*g* for 10min and the pH of the supernatant adjusted to 6.0 with 1M-HCl. DEAE-Sephadex A-50 (250mg), which bound acidic proteins, was added with stirring. The Sephadex was subsequently collected by sedimentation at 3000*g* for 5min and redispersed in the above solution (pH6.0). Sedimentation and resuspension steps were repeated three times more to remove unbound material. The bound proteins were recovered from the Sephadex by sedimentation in the same solution containing 0.6M-NaCl. Sp-Sephadex C-50 (250mg) was added to the supernatant, and this was then dialysed against 90mM-NaCl/1mM-EDTA/6M-urea/10mM-sodium acetate buffer, pH5.0. The Sephadex was removed by sedimentation at 3000*g* for 5min and the supernatant dialysed against 1mM-$CaCl_2$/6M-urea/15mM-2-mercaptoethanol/20mM-Tris buffer, pH7.8. The solution was applied to an affinity column (2.5cm × 7.5cm) containing rabbit troponin I covalently bound to Sepharose 4B (Head *et al.*, 1977*b*) equilibrated with the above solution. Unbound material was eluted with 500ml of this buffer, and calmodulin then eluted with the same solution but with 5mM-EGTA replacing the $CaCl_2$. Fractions containing the protein were dialysed against 15mM-2-mercaptoethanol/20mM-Tris buffer, pH 7.8, and freeze-dried. This procedure yields approx. 6–8μg of calmodulin.

Preparation of vertebrate proteins

Rabbit actin free of tropomyosin contamination was prepared by the method of Straub (1942) by using the modification described by Drabikowski & Gergely (1964). Rabbit tropomyosin was extracted and purified by the method described by Bailey (1948) as modified by Lehman & Szent-Györgyi (1972). Ca^{2+}-insensitive rabbit myosin was prepared from longissimus dorsi muscle as described by Szent-Györgyi (1951) and Mommaerts & Parrish (1951). Rabbit troponin C was prepared from whole troponin as described by Greaser & Gergely (1971). Bovine brain cyclic AMP phosphodiesterase and calmodulin were prepared by the method of Head *et al.* (1979).

Preparation of antisera to scallop troponin I and to scallop troponin C

Preparations of scallop troponin I are frequently contaminated with small amounts of a high-molecular-weight protein, and scallop troponin C is sometimes contaminated with traces of tropomyosin. To ensure the purity of the troponin I and troponin C used for antibody production, disc-gel electrophoresis was performed on preparations of each protein. The appropriate band positions were determined, by scanning at 280nm or by Coomassie Brilliant Blue R staining a sample gel, and the band areas then sliced out of unstained gels. 10% (w/v) polyacrylamide/SDS gels were used for electrophoresis of troponin I preparations and alkaline 1M-urea/8% acrylamide gels for troponin C preparations (see under 'Analysis' for electrophoretic methods). A sample (70–100μg) of the appropriate protein, embedded in polyacrylamide from 12–17 gels, was homogenized in a glass tissue grinder in approx. 10ml of 145mM-NaCl and the entire suspension injected into a rabbit subcutaneously at several sites. This procedure was repeated and the

rabbit re-injected once each week for 6 weeks. In the first two injections, the suspension was emulsified with 3ml of Freund's complete adjuvant. After 7 weeks, blood was taken from an ear vein; the blood was allowed to clot at 4°C for 12–16h and serum separated by sedimentation at 3000*g* for 20min.

Analysis

Electrophoresis. SDS/polyacrylamide-disc-gel electrophoresis was performed as described by Weber & Osborn (1969). The gels were stained and destained as described by Szent-Györgyi *et al.* (1973). Densitometric scanning (at 640nm) of Fast Green (Fisher-FCF)-stained gels was performed on a Gilford 240 spectrophotometer with a linear transport adaptor and a Beckman DU monochromator.

Relative dye-binding constants of purified proteins were measured as described previously (Lehman *et al.*, 1976). The relative stain intensities of different proteins contained in scallop myofibrils and thin filaments were determined similarly from gel scans.

In myofibrillar preparations, resolution of troponin C (18000 daltons) from myosin light chains (17000 daltons) was most successful with 6-cm-long SDS/12.5% polyacrylamide gels (run for 4h at 50V), and this system was used to determine relative stain intensities of scallop actin, tropomyosin and troponin C bands. (Some overlap of troponin C and light-chain peaks occurs in this system; determination of the shape of light-chain peaks, on gels of purified scallop myosin, shows it is reasonable to extrapolate the trailing edge of the light-chain peak to the baseline, the troponin C peak being isolated for determination.) On 12.5% polyacrylamide gels the troponin I band is diffuse and not readily resolved; however, this protein can be easily distinguished by using 10% polyacrylamide gels (run for 2.5h at 50V), and this system was used to determine the relative intensity of troponin I and tropomyosin.

A second electrophoretic technique, alkaline-gel electrophoresis, was used in many of our studies with gels containing either 1M- or 6M-urea and 8% polyacrylamide, as described by Head & Perry (1974). Samples electrophoresed (0.5h at 200V) in the presence of Ca^{2+} contained 5mM-$CaCl_2$; those in the absence of Ca^{2+} contained 5mM-EGTA.

Protein determination. Protein concentration, used in determining dye-binding constants, was measured by micro-Nesslerization, with $(NH_4)_2SO_4$ standards (Strauch, 1965) and assuming that protein samples contain 16% N. Since the dye-binding constants of scallop and rabbit troponins C were found to be the same, the protein concentration of scallop troponin C used in Ca^{2+}-binding and enzymic studies could be determined by densitometric gel scanning with rabbit troponin C standards. For general purposes, protein concentration was determined by the method of Lowry *et al.* (1951).

Assays. The actomyosin Mg-ATPase was measured by the pH-stat method described previously (Szent-Györgyi *et al.*, 1971; Goldberg & Lehman, 1978). Cyclic AMP phosphodiesterase activity was measured by the method of Boudreau & Drummond (1975). Ca^{2+} binding was determined by using equilibrium dialysis, in the presence of 0.1mM-$CaCl_2$ and 1mM-$MgCl_2$, the method of Head *et al.* (1979) being used.

Immunodiffusion. Antisera were tested for reaction with antigens by the Ouchterlony (1953) immunodiffusion method. 5cm-square glass plates were filled with 5ml of 1% agarose dissolved in 145mM-NaCl/0.5mM-NaN_3/10mM-sodium phosphate buffer, pH7.0, and wells punched out with a spacing of 0.5cm. Antisera (30–60μl) and various amounts of antigens were placed into the wells. Precipitin arcs developed within 2 days. Plates were then washed with 0.15M-NaCl/10mM-sodium phosphate buffer, pH7.0, for 12–16h, followed by a 2h wash with distilled water. The plates were air-dried and finally stained with Coomassie Brilliant Blue R.

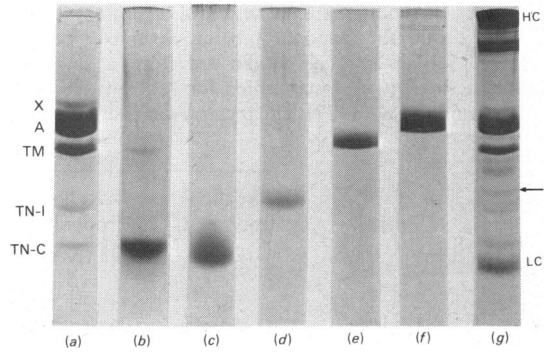

Fig. 1. *SDS/polyacrylamide-gel electrophoresis of various preparations used in the present study*

Gels (10% acrylamide) were stained with Coomassie Brilliant Blue R. (*a*) Native thin filaments (48μg); (*b*) scallop troponin C (8μg); (*c*) king-crab troponin C; (*d*) scallop troponin I (2μg); (*e*) scallop tropomyosin (9μg); (*f*) scallop actin (20μg); (*g*) washed scallop myofibrils (75μg). The arrow next to gel (*g*) indicates a protein that migrates slightly slower than troponin I. This protein can be removed if 1% Triton X-100 is included in the myofibril-wash solution, without affecting measurements of relative stain intensity of other bands. Abbreviations used: X, a protein that may be troponin T; A, actin; TM, tropomyosin; TN-I, troponin I; TN-C, troponin C; HC, myosin heavy chain; LC, myosin light chain.

Indirect immunofluorescence microscopy. Stretched adductor muscles, attached to the valves, were prepared from the bay scallops as described by Kendrick-Jones *et al.* (1970) and were immersed in 50% (v/v) glycerol/100mM-NaCl/1mM-$MgCl_2$/0.2mM-EGTA/10mM-sodium phosphate buffer, pH 7.0, for 12–16h. Bundles of muscle fibres were teased off, homogenized and washed by sedimentation and redispersion with the same solution, but free of glycerol. A drop containing these myofibrils was placed on a glass slide and fixed with 3.7% formaldehyde. The slides were washed with 0.15M-NaCl/10mM-sodium phosphate buffer, pH 7.0. A sample (50μl) of antiserum was then placed on the myofibrils adhering to the slide, and excess antiserum was washed off as above. A portion (15μl) of fluorescein-conjugated goat anti-(rabbit IgG) serum (0.5mg/ml: Cappel Laboratories, Cochranville, PA, U.S.A.) was placed on the myofibrils and excess anti-IgG then washed off. The myofibrils were examined with a Zeiss microscope with phase-contrast and epifluorescence optics.

Results

Scallop troponin C-like protein

We have isolated, from native scallop thin filaments, an 18000-dalton component that by analogy to the vertebrate troponin subunit may represent scallop troponin C. The protein migrates as a single band on SDS/polyacrylamide gels (Fig. 1*b*) and also on alkaline 1M- and 6M-urea/polyacrylamide gels (Figs. 2*a* and 2*d*).

Although rabbit skeletal-muscle troponin C binds four Ca^{2+} ions per molecule, our preliminary Ca^{2+}-binding studies indicate that the scallop protein binds only one Ca^{2+} ion per molecule (measured in 0.1mM-$CaCl_2$/1mM-$MgCl_2$/100mM-NaCl/10mM-imidazole buffer, pH 7.0).

The amino-acid compositions of rabbit, king crab (*Limulus*) and scallop troponin C are shown in Table 1. Whereas the three proteins have a high phenylalanine/tyrosine ratio, characteristic of troponin C, the (Glx + Asx)/(Lys + Arg) ratio, high in rabbit and king crab, is lower in scallop troponin

Table 1. *Amino-acid compositions of troponin C-like proteins and scallop troponin I*

Values are mol of amino acid/mol of protein in 24h hydrolysates, mol.wts. of 18000 for troponin C-like proteins and 23000 for troponin I being assumed. Values for scallop troponin C are averages for three preparations; those for scallop calmodulin are from a single preparation; for king-crab troponin C and scallop troponin-I, averages of two preparations; for rabbit troponin C and bovine calmodulin, from sequence data (Collins, 1974; Vanaman *et al.*, 1977). The tryptophan content was not measured.

		Content					
Amino acid	Protein ...	Scallop troponin C	Rabbit troponin C	King-crab troponin C	Scallop calmodulin	Bovine brain calmodulin	Scallop troponin I
Asx		23.0	23 (3 Asn)	23.1	22.4	23	22.5
Thr		7.1	5	9.9	14.4	12	7.1
Ser		8.7	7	8.5	8.1	4	13.2
Glx		26.9	31 (5 Gln)	31.0	25.3	27	31.0
Pro		2.2	1	0.7	3.8	2	6.8
Gly		11.8	13	12.5	16.9	11	14.7
Ala		10.8	13	11.4	13.1	11	20.6
Val		10.4	7	6.3	8.3	7	10.2
Cys		1.1	1	0.7	—	0	2.9
Met		3.9	10	6.8	2.0	9	2.3
Ile		7.0	10	8.1	8.4	8	6.6
Leu		16.0	9	15.6	11.5	9	18.1
Tyr		1.6	2	2.1	0.8	2	2.3
Phe		7.3	10	8.7	10.1	8	6.6
Lys		16.2	9	6.3	9.2	8	32.2
His		0.7	1	0.8	1.9	1	3.4
Arg		6.1	7	7.7	8.4	6	8.1
$\frac{Asx + Glx}{Lys + Arg}$		2.2	3.4	3.9	2.7	3.6	1.3
$\frac{Asp + Glu}{Lys + Arg}$			2.9				

C, suggesting that the scallop troponin C-like protein to be less acidic. Although we have not measured the absolute amounts of aspartate and glutamate with respect to their amines, in scallop troponin C, we point out that even if there were no amine forms, which would be the most 'acidic' possibility, the scallop troponin C would still be less acidic than the rabbit skeletal-muscle protein [Table 1; compare (Glx + Asx)/(Lys + Arg) for scallop troponin C with (Glu + Asp)/Lys + Arg) for rabbit troponin C].

Electrophoresis carried out under alkaline conditions gives additional insight into the similarities and differences between rabbit and scallop troponin C. Both proteins migrate at the same rate on alkaline 1 M-urea/polyacrylamide gels; however, on alkaline 6 M-urea/polyacrylamide gels the electrophoretic mobility of rabbit troponin C is greater (Fig. 2). Their co-migration in 1 M-urea probably results from similar conformation and size, masking any charge differences. The difference in migration in 6 M-urea presumably reflects either dissimilarity in number or exposure of charged residues, as would be expected of proteins with differing acidities, or differences in susceptibility to urea denaturation and associated changes in conformation.

Electrophoresis of 1 M-urea extracts of scallop myofibrils and thin filaments on alkaline 1 M-urea/polyacrylamide gels yields different band patterns if Ca^{2+} is present or absent. A rapidly migrating band, with the mobility of scallop troponin C, is found in extracts containing EGTA, whereas this band is substantially weaker in intensity when these extracts contain Ca^{2+} (Fig. 3). In contrast, the same electrophoretic pattern is obtained for molluscan muscle extracts in 6 M-urea, whether Ca^{2+} is present or not (Head *et al.*, 1977*b*; also confirmed in the present study).

We showed previously that scallop muscle contains a troponin I-like component that causes a Ca^{2+}-independent inhibition of vertebrate actomyosin ATPase (Goldberg & Lehman, 1978; Table 2*b* of the present paper). In the present study we have found that addition of scallop troponin C-like protein to the troponin I-inhibited ATPase relieves this inhibition without conferring a Ca^{2+}-dependence (Table 2*c*). This effect is analogous to that of vertebrate troponin C and troponin I on actomyosin assayed in the absence of troponin T. We have not, as yet, succeeded in purifying the scallop 'troponin T-like' protein and therefore have not been able to reconstitute and assay the entire troponin-like complex.

Scallop calmodulin

We have also isolated another 18000-dalton protein from scallop muscle by using vertebrate troponin I–Sepharose 4B affinity columns. The protein forms a stable complex with vertebrate troponin I in 6 M-urea in the presence of Ca^{2+} and dissociates from the troponin I in EGTA (Fig. 4). Such behaviour is an attribute of both vertebrate troponin C and the structurally related protein, calmodulin (Head *et al.*, 1979). We have been able to isolate the protein from myofibril extracts by

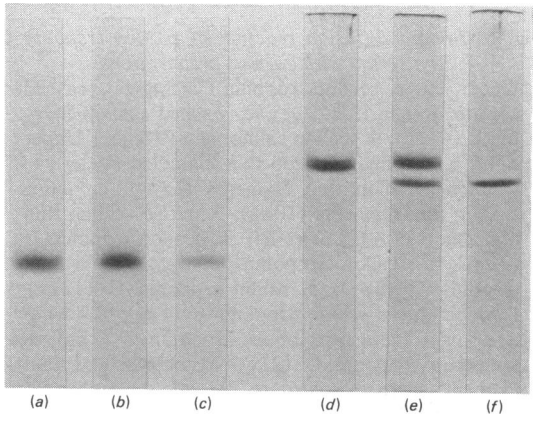

Fig. 2. *Alkaline-gel electrophoresis of scallop and rabbit troponins*

Gels (*a*), (*b*) and (*c*) contain 1 M-urea; gels (*d*), (*e*) and (*f*) contain 6 M-urea. (*a*) and (*d*), scallop troponin C (7 μg); (*c*) and (*f*), rabbit troponin C (5 μg); (*b*) and (*e*), scallop and rabbit troponins C (7 and 5 μg); Coomassie Brilliant Blue R staining was used. All samples contained EGTA.

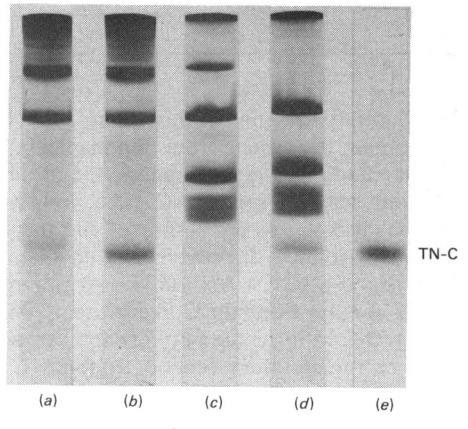

Fig. 3. *Effect of Ca^{2+} on the alkaline 1 M-urea/polyacrylamide-gel-electrophoretic pattern*

(*a*) and (*b*), synthetic scallop thin filaments (50 μg); (*c*) and (*d*), washed scallop myofibrils (150 μg); (*e*) scallop troponin C (7 μg). Samples in gels (*a*) and (*c*) contained Ca^{2+}; samples in gels (*b*), (*d*) and (*e*) contained EGTA; Coomassie Brilliant Blue R staining was used. Abbreviation used: TN-C, troponin C.

Table 2. *Effect of scallop troponin I and troponin C on rabbit actomyosin ATPase*

ATPase activity was assayed in 30 mM-NaCl/3 mM-$MgCl_2$/0.7 mM-ATP containing either 0.1 mM-EGTA (+EGTA) or 0.1 mM-EGTA/0.2 mM-$CaCl_2$ (+Ca^{2+}); activity is expressed in μmol of ATP cleaved/min per mg of rabbit myosin.

Sample assayed	Mg-ATPase activity +EGTA	Mg-ATPase activity +Ca^{2+}
(*a*) Rabbit tropomyosin (0.08 mg) + rabbit actin (0.17 mg) + rabbit myosin (0.5 mg)	0.41	0.42
(*b*) Scallop troponin I (0.08 mg) + rabbit tropomyosin (0.08 mg) + rabbit actin (0.17 mg) + rabbit myosin (0.5 mg)	0.16	0.17
(*c*) Scallop troponin C (0.08 mg) + scallop troponin I (0.08 mg) + rabbit tropomyosin (0.08 mg) + rabbit actin (0.17 mg) + rabbit myosin (0.5 mg)	0.41	0.36

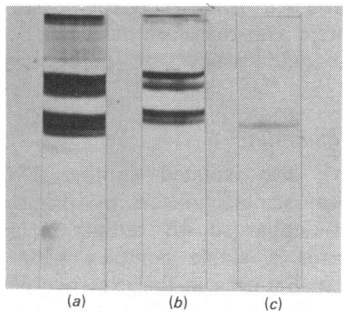

Fig. 4. *Alkaline 6 M-urea/polyacrylamide-gel electrophoresis of scallop calmodulin preparations*
(*a*) Scallop extract before application on vertebrate troponin I affinity column; (*b*) material eluted from column in the presence of Ca^{2+}; (*c*) protein (calmodulin) eluted with EGTA; see the Materials and Methods section for details.

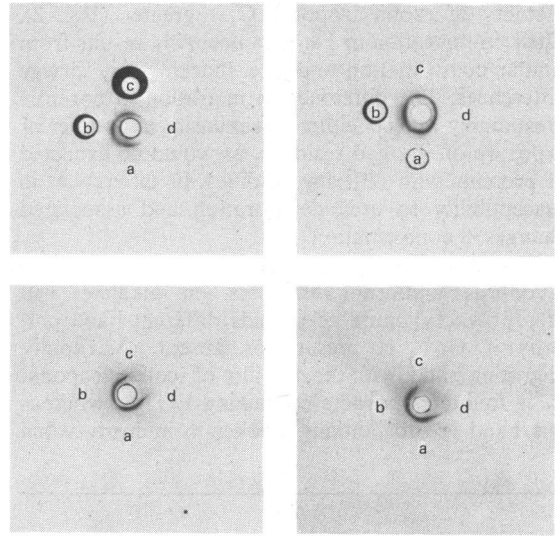

Fig. 5. *Immunodiffusion reaction of scallop troponin C antiserum with various preparations*
Upper left: a, scallop troponin C (1 μg); b, washed scallop myofibrils (20 μg); c, washed scallop myofibrils (60 μg); d, scallop calmodulin (0.03 μg). Upper right: a, synthetic scallop thin filaments (10 μg); b, synthetic scallop thin filaments (25 μg); c, native scallop thin filaments (10 μg); d, native scallop thin filaments (25 μg). Lower left: a, scallop troponin C (2 μg); b, scallop troponin C (4 μg); c, rabbit troponin C (6 μg); d, rabbit troponin C (12 μg). Lower right: a, scallop troponin C (1 μg); b, scallop troponin C (2 μg); c, lobster troponin C (2 μg); d, king-crab troponin C (2 μg). Myofibrils and thin filaments were dissolved in 8 M-urea before application to wells.

using the affinity-column method, but have been unable to obtain the protein from thin filaments with the same method. This result suggests the protein is distinct from scallop troponin C. The amino-acid composition also differs from that of the troponin C-like protein isolated directly from thin filaments [Table 1; e.g. the (Glx + Asx)/(Lys + Arg) ratio is higher than for the troponin C-like protein]. Moreover, it migrates with the mobility of rabbit troponin C and more rapidly than scallop troponin C, on alkaline 6 M-urea/polyacrylamide gels. Immunodiffusion studies also show that antisera prepared against scallop troponin C-like protein do not cross-react with this protein (Fig. 5).

Studies of the effect of the protein on phosphodiesterase activity lead us to believe it may represent scallop muscle calmodulin. Enzyme assays show the protein to activate bovine brain cyclic AMP phosphodiesterase to the same extent as equal amounts of bovine brain calmodulin and to render the enzyme's activity Ca^{2+}-dependent (Fig. 6). Control studies show no enhancement of activity when scallop troponin C is substituted for calmodulin.

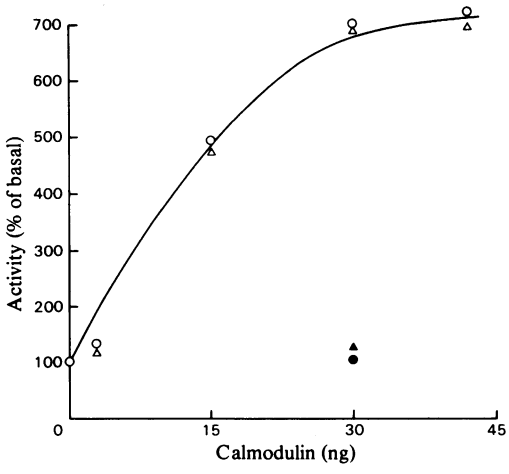

Fig. 6. *Activation of brain cyclic AMP phosphodiesterase by calmodulin*

O, Activation by brain calmodulin; △, by scallop calmodulin. The basal enzyme activity was 320μmol/min per mg. Open symbols denote samples with Ca^{2+}; closed symbols denote samples with EGTA.

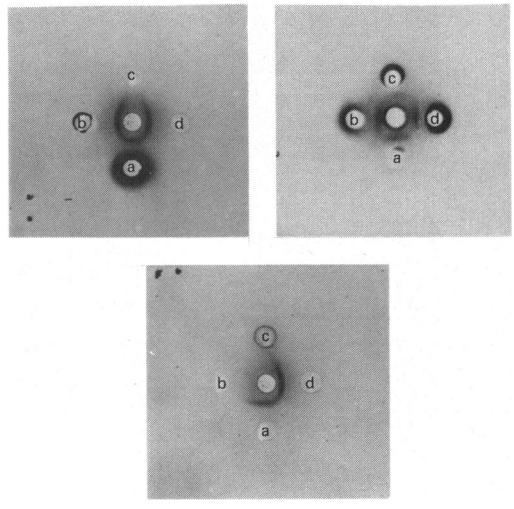

Fig. 7. *Immunodiffusion reaction of scallop troponin I antiserum with various preparations*

Upper left: a, washed scallop myofibrils (9μg); b, scallop troponin I (2μg); c, crushed blank SDS/polyacrylamide-gel slice; d, scallop troponin I (2μg in 1% SDS). Upper right: a, synthetic scallop thin filaments (10μg); b, synthetic scallop thin filaments (30μg); c, native scallop thin filaments (17μg); d, native scallop thin filaments (50μg). Lower middle: a, scallop troponin I (1μg); b, 0.9% NaCl; c, rabbit troponin I (2μg); d, scallop troponin I (2μg).

Scallop troponin I

The purification and partial characterization of scallop troponin I was described previously (Goldberg & Lehman, 1978). In the present study we have determined its amino-acid composition (Table 1), and, in common with vertebrate troponin I, we find it to be a relatively basic protein.

Localization of troponin components

We have prepared rabbit antisera against purified scallop troponin C and purified scallop troponin I. Immunodiffusion studies show the formation of a single precipitin arc when anti-(scallop troponin C) antiserum is diffused against scallop troponin C or against extracts of scallop thin filaments or myofibrils. No reaction is observed with scallop actin, tropomyosin or troponin I, i.e. the other components of the thin filaments. The antiserum also does not cross-react with troponin C from rabbit, king crab or lobster (*Homarus*) (Fig. 5). Rabbit anti-(scallop troponin I) also shows specificity for the troponin I, forming a single precipitin line against purified scallop troponin I and against extracts of scallop thin filaments or myofibrils (Fig. 7). It shows no cross-reaction with scallop actin, tropomyosin or troponin C, nor with rabbit or lobster troponin I.

The technique of indirect immunofluorescence staining was used to localize troponin C and troponin I in scallop myofibrils. In Fig. 8, paired phase-contrast and fluoresence micrographs show scallop myofibrils stained with antiserum against either scallop troponin C or scallop troponin I and counterstained with fluorescein-labelled goat anti-(rabbit IgG). All myofibrils observed show fluorescent staining of the I-bands.

Quantification of thin-filament components

To ascertain if the molar ratios of the scallop thin-filament components are similar to the stoichiometry found in vertebrate muscle, determination of the weight ratios of the components in the intact scallop thin filament and their molecular weights is needed. The molecular weights of scallop actin, tropomyosin, troponin C and troponin I have been defined previously (Lehman & Goldberg, 1978). In the present study we have determined the weight ratios of these proteins by using quantitative polyacrylamide-gel densitometry, the stain intensity ratios of the respective electrophoretic bands being measured. Since each protein binds a different amount of dye per unit weight, we have purified each protein (Fig. 1) and determined dye-binding constants for each (Table 3).

Actin, tropomyosin and troponin C bands can be resolved densitometrically on SDS/polyacrylamide

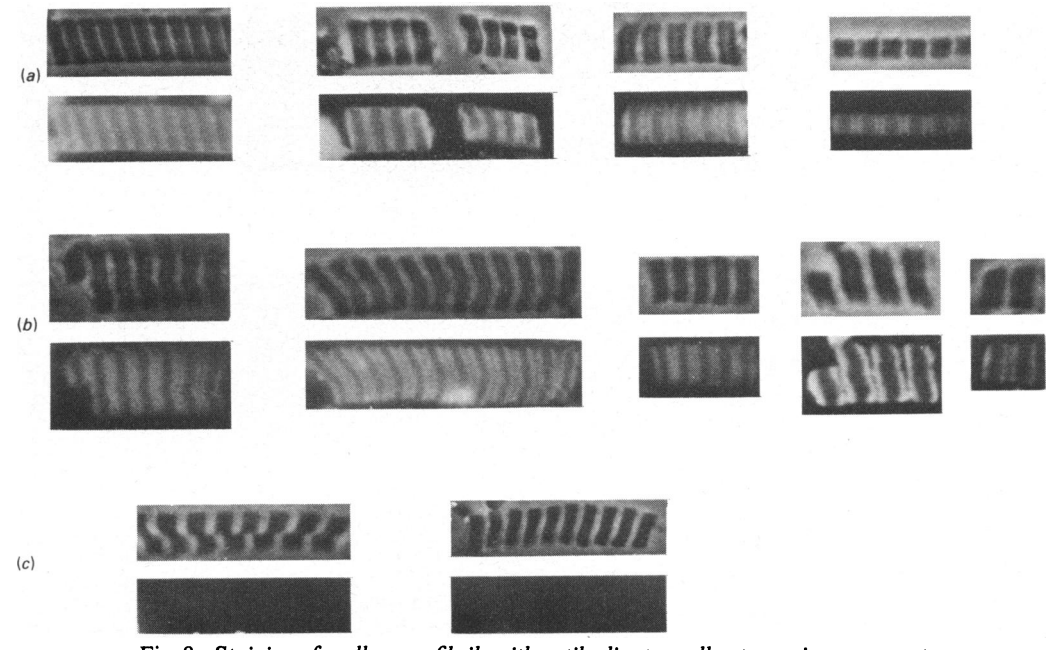

Fig. 8. *Staining of scallop myofibrils with antibodies to scallop troponin components*
Upper micrograph of each pair, phase-contrast optics; lower micrograph, fluorescence optics. (*a*) Myofibrils stained with troponin C antibodies and counterstained with fluorescein-labelled anti-IgG;(*b*) myofibrils stained with troponin I) antibodies and counterstained with fluorescein-labelled anti-IgG; (*c*) controls in which antiserum adsorbed with excess antigen before staining; pair on left, adsorption with troponin C; pair on right, adsorption with troponin I. 'I-bands' appear broader in fluorescence than in phase optics, since thin filaments in both I-bands and in lateral edges of A-bands are stained.

Table 3. *Stoichiometry of scallop troponin C*
Averages ± S.D. of relative stain intensities of actin, tropomyosin and troponin C bands from six different unwashed and washed myofibril preparations and five different native-thin-filament preparations. Dye-binding constants, relative to tropomyosin, from two preparations of each protein are given, with individual values in parentheses. From these values and the proteins' molecular weights, molar ratios have been calculated for each of the different preparations.

Sample	Protein	Relative-stain-intensity ratio	Dye-binding constant	Molecular weight	Molar ratio
Unwashed myofibrils	Actin	3.4 ± 0.12	0.79 (0.79, 0.79)	42000	7.2
Washed myofibrils		3.2 ± 0.14			6.8
Thin filaments		3.7 ± 0.27			7.9
Unwashed myofibrils	Tropomyosin	1.0	1.0	70000	1.0
Washed myofibrils		1.0			1.0
Thin filaments		1.0			1.0
Unwashed myofibrils	Troponin C	0.14 ± 0.012	0.57 (0.55, 0.59)	18000	1.0
Washed myofibrils		0.15 ± 0.015			0.96
Thin filaments		0.18 ± 0.035			1.2

gels of unwashed and washed scallop myofibrils and on SDS/polyacrylamide gels of native thin filaments. Relative stain intensities were therefore determined for these proteins in each of the three types of preparations (Table 3). On the basis of these values, the molar ratio actin/tropomyosin/troponin C was calculated to be 7 : 1 : 1.

In unwashed myofibrils we were unable to measure scallop troponin I on SDS/polyacrylamide gels, because of the closely migrating so-called 'soluble Ca^{2+}-binding protein' identified by Lehman & Szent-Györgyi (1975). This protein is readily removed by washing, and we were therefore able to make a determination of troponin I content in the

washed myofibril preparation by using the SDS/polyacrylamide-gel system. A value of 0.6 ± 0.04 mol of troponin I/mol of tropomyosin was calculated (four different myofibril preparations analysed; the relative dye-binding constant of troponin I/tropomyosin is 1.0). Determinations of troponin I in native thin filaments gave inconsistent values with considerable variation, ranging from 0.4–0.6 mol of troponin I/mol of tropomyosin.

Discussion

In a previous investigation (Goldberg & Lehman, 1978) we isolated proteins from the bay scallop that bind to actin and confer Ca^{2+}-dependency on vertebrate actomyosin ATPase. The proteins, also found on native thin filaments, consist of tropomyosin and three additional components (mol.wts. 18000, 23000, 55000), possibly troponin subunits. The conclusion that the latter proteins may represent troponin subunits was supported by results on the purified 23000-dalton component, which behaves like vertebrate troponin I and inhibits vertebrate actomyosin ATPase. The component also interacts with vertebrate troponin C and troponin T, and the hybrid complex confers a Ca^{2+}-dependence on actomyosin ATPase. We have continued this work and, in the present study, have purified the 18000-dalton component, which appears to be analogous to vertebrate troponin C. It is an acidic protein that binds Ca^{2+} and relieves the inhibition of actomyosin ATPase by troponin I.

Although scallop troponin C is an acidic protein, it appears to be somewhat less acidic than the troponin C in higher organisms. Since the molluscan phylum developed earlier in evolution than the vertebrate and arthropod phyla, the molluscan troponin C may be more closely related to an archetypal protein. Knowledge of the amino-acid sequence of scallop troponin C would therefore be of great interest in understanding the evolution of the protein.

We have found that the apparently ubiquitous protein calmodulin also exists in scallop muscle. Calmodulin has been described in vertebrate smooth and striated muscles (Head *et al.*, 1977*a*; Dabrowska *et al.*, 1978; Yagi *et al.*, 1978; Nairn & Perry, 1979) and has been implicated as an activator of myosin light-chain kinases in these muscles (Dabrowska *et al.*, 1978; Yagi *et al.*, 1978; Nairn & Perry, 1979). In the present study we have been principally concerned with ensuring that scallop calmodulin and scallop troponin C are distinct proteins and have not attempted to evaluate the functional role of calmodulin in scallop muscles.

Vertebrate troponin I and troponin C form a stable complex in 6 M-urea and in the presence of Ca^{2+}, which dissociates in EGTA. Since this property can be easily demonstrated by alkaline-gel electrophoresis and is also manifested by crude extracts containing troponin C and troponin I, it has been used as an indicator for the presence of vertebrate troponin-like proteins (Head & Perry, 1974). Similar experiments carried out on molluscan and other invertebrate muscles failed to detect Ca^{2+}-dependent complex-formation in 6 M-urea (Head *et al.*, 1977*b*). In molluscs, such complexes are apparently not stable at this urea concentration; however, in 1 M-urea, gel electrophoresis shows the troponin C-like protein to be associated with other thin-filament proteins in the presence of Ca^{2+} and dissociated in EGTA. Hence, alkaline 1 M-urea/polyacrylamide-gel electrophoresis may be a simple technique, useful for testing the presence of troponin in invertebrates. Preliminary experiments on lobster muscle show the same behaviour as observed in scallop.

Quantitative SDS/polyacrylamide-gel electrophoresis has been used to determine the stoichiometry of troponin-like subunits in scallop preparations, purified proteins being used as standards. We find a molar ratio of 7:1:1 for actin/tropomyosin/troponin C-like protein in preparations of untreated myofibrils, washed myofibrils and isolated native thin filaments. The consistency of this ratio in the three types of preparation indicates that the troponin C-like protein is not washed from the thin filaments during preparation. It also suggests that the procedure used for isolating thin filaments does not enrich for a specific population containing troponin. We believe that insufficient amounts of calmodulin are present to affect significantly the measurement of troponin C in the myofibril preparations. In thin-filament preparations we did not detect any calmodulin with the 6 M-urea/polyacrylamide-gel-electrophoresis system, which separates scallop troponin C from calmodulin.

If we assume the value for troponin C to be a measure of the entire troponin-like complex, then the data suggest a model very similar to that of vertebrate striated muscle, where there is also one tropomyosin and one troponin complex interacting with seven actin monomers along the thin filament. However, measurements of scallop troponin I content are more difficult to interpret. The quantity of troponin I-like protein could not be determined with accuracy in untreated myofibrils because of interfering proteins on SDS/polyacrylamide gels; in washed myofibrils, the amount was consistently found to be 0.6 mol/mol of tropomyosin. The value is lower than that found for scallop troponin C-like protein and lower than that expected from the vertebrate model. At present we have no satisfactory explanation for the non-integral stoichiometry. It is possible that proteolysis or extraction during washing may have decreased the amount of

troponin I present. Such effects might also explain the variability in results obtained by using thin-filament preparations.

Localization studies using indirect immunofluorescence staining with antibodies prepared against scallop troponin I and troponin C show staining of I-bands on all scallop myofibrils examined, indicating the presence of these proteins on the thin filament in the intact myofibril.

We believe that the results of our investigations, taken together, provide strong evidence for the existence of a thin-filament-linked regulatory system in the striated muscle of the bay scallop.

This investigation was supported by grants (nos. AM 17062 and AM 18207) from the National Institutes of Health and a grant (no. 13-503-778) from the Muscular Dystrophy Associations of America. We thank Dr. Judith Saide for advice on immunohistochemistry and the use of her microscope, Mr. James Munroe for laboratory assistance, Dr. Carl Franzblau for making his amino-acid analyser available, and Mr. George Crombie for performing the analyses.

References

Bailey, K. (1948) *Biochem. J.* **43**, 271–279

Boudreau, R. J. & Drummond, G. I. (1975) *Anal. Biochem.* **63**, 388–399

Collins, J. H. (1974) *Biochem. Biophys. Res. Commun.* **58**, 301–308

Dabrowska, R. J., Sherry, J. M. F., Aramatorio, D. K. & Hartshorne, D. S. (1978) *Biochemistry* **17**, 253–258

Drabikowski, W. & Gergely, J. (1964) in *Biochemistry of Muscle Contraction* (Gergely, J., ed.), pp. 125–131, Little, Brown and Co., Boston

Goldberg, A. & Lehman, W. (1978) *Biochem. J.* **171**, 413–418

Grand, J. A., Perry, S. V. & Weeks, R. A. (1979) *Biochem. J.* **177**, 521–529

Greaser, M. L. & Gergely, J. (1971) *J. Biol. Chem.* **246**, 4226–4233

Head, J. F. & Perry, S. V. (1974) *Biochem. J.* **137**, 145–154

Head, J. F., Mader, S. & Kaminer, B. (1977*a*) in *Calcium-Binding Proteins and Calcium Function* (Wasserman, R. H., Corradino, R. A., Kretsinger, R. H., MacLennan, D. H. & Siegel, F. L., eds.), pp. 275–277, Elsevier/North-Holland, Amsterdam

Head, J. F., Weeks, R. A. & Perry, S. V. (1977*b*) *Biochem. J.* **137**, 465–471

Head, J. F., Mader, S. & Kaminer, B. (1979) *J. Cell Biol.* **80**, 211–218

Kendrick-Jones, J., Lehman, W. & Szent-Györgyi, A. G. (1970) *J. Mol. Biol.* **54**, 313–326

Konno, K. (1978) *J. Biochem. (Tokyo)* **84**, 1431–1440

Lehman, W. (1977) *Biochem. J.* **163**, 291–296

Lehman, W. & Szent-Györgyi, A. G. (1972) *J. Gen. Physiol.* **59**, 375–387

Lehman, W. & Szent-Györgyi, A. G. (1975) *J. Gen. Physiol.* **66**, 1–30

Lehman, W., Kendrick-Jones, J. & Szent-Györgyi, A. G. (1972) *Cold Spring Harbor Symp. Quant. Biol.* **37**, 319–330

Lehman, W., Regenstein, J. M. & Ransom, A. L. (1976) *Biochim. Biophys. Acta* **434**, 215–222

Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275

Mommaerts, W. F. H. M. & Parrish, R. G. (1951) *J. Biol. Chem.* **188**, 545–552

Nairn, A. C. & Perry, S. V. (1979) *Biochem. J.* **179**, 89–97

Ouchterlony, O. (1953) *Acta Pathol. Microbiol. Scand.* **32**, 231–240

Spudich, J. A. & Watt, S. (1971) *J. Biol. Chem.* **246**, 4866–4871

Straub, F. B. (1942) *Stud. Inst. Med. Chem. Univ. Szeged* **2**, 3–16

Strauch, L. (1965) *Z. Klin. Chem. Klin. Biochem.* **3**, 165–167

Szent-Györgyi, A. G. (1951) *Chemistry of Muscular Contraction*, 2nd edn., pp. 146–148, Academic Press, London and New York

Szent-Györgyi, A. G. (1976) *Symp. Soc. Exp. Biol.* **30**, 335–347

Szent-Györgyi, A. G., Cohen, C. & Kendrick-Jones, J. (1971) *J. Mol. Biol.* **56**, 239–258

Szent-Györgyi, A. G., Szentkiralyi, E. M. & Kendrick-Jones, J. (1973) *J. Mol. Biol.* **74**, 179–203

Tsuchiya, T., Kaneko, T. & Matsumoto, J. J. (1978) *J. Biochem. (Tokyo)* **83**, 1191–1193

Vanaman, T. C., Sharief, F. & Watterson, D. M. (1977) in *Calcium-Binding Proteins and Calcium Function* (Wasserman, R. A., Corradino, E., Kretsinger, R. H., MacLennan, D. H. & Siegel, F. L., eds.), pp. 107–116, Elsevier/North-Holland, Amsterdam

Weber, K. & Osborn, M. (1969) *J. Biol. Chem.* **244**, 4406–4412

Yagi, K., Yazawa, M., Kakiuchi, S., Oshima, M. & Venishi, K. (1978) *J. Biol. Chem.* **253**, 1338–1340