References

Anderson, G., and Graves, G. (1973), *Biochemistry 12,* 1895.
Baker, B. R. (1967), Design of Active-Site-Directed Irreversible Enzyme Inhibitors, New York, N.Y., Wiley.
Baker, B. R., Lee, W. W., and Tong, E. (1962), *J. Theor. Biol. 3,* 459-483.
Brunswick, D. J., and Cooperman, B. S. (1971), *Proc. Nat. Acad. Sci. U.S. 68,* 1801.
Clark, C. F., and Yielding, K. L. (1971), *Arch. Biochem. Biophys. 143,* 158.
Colman, R. F., and Frieden, C. (1966a), *J. Biol. Chem. 241,* 3652-3660.
Colman, R. F., and Frieden, C. (1966b), *J. Biol. Chem. 241,* 3661.
Colman, R. F., and Frieden, C. (1966c), *Biochem. Biophys. Res. Commun. 22,* 100.
Cross, D. G., and Fisher, H. F. (1970), *J. Biol. Chem. 245,* 2612-2621.
diPrisco, G. (1971), *Biochemistry 10,* 585.
Fisher, H. F. (1973), *Advan. Enzymol. 39,* 369-417.
Frieden, C. (1959), *J. Biol. Chem. 234,* 809, 815.
Frieden, C. (1963), *J. Biol. Chem. 238,* 3286-3299.
Goldin, B. R., and Frieden, C. (1971), *Biochemistry 10,* 3527-3534.
Goldin, B. R., and Frieden, C. (1972a), *Curr. Top. Cell. Regul. 4,* 77-117.
Goldin, B. R., and Frieden, C. (1972b), *J. Biol. Chem. 247,* 2139-2144.
Hulla, F. W., and Fasold, H. (1972), *Biochemistry 11,* 1056.
Koberstein, R., Krause, J., and Sund, H. (1973), *Eur. J. Biochem. 40,* 543-548.
Koberstein, R., and Sund, H. (1973), *Eur. J. Biochem. 36,* 545-552.
Krause, J., Buhner, M., and Sund, H. (1974), *Eur. J. Biochem. 41,* 593-602.
Olson, J. A., and Anfinsen, C. B. (1952), *J. Biol. Chem. 197,* 67.
Pal, P. K., Wechter, W. J., and Colman, R. F. (1974), Abstracts of the 168th National Meeting of the American Chemical Society, Atlantic City, N.J.
Pantaloni, D., and Dessen, P. (1969), *Eur. J. Biochem. 11,* 510.
Pantaloni, D., and Lécuyer, B. (1973), *Eur. J. Biochem. 40,* 381-401.
Poulos, T. L., and Price, P. A. (1974), *J. Biol. Chem. 249,* 1453.
Price, N. C., and Radda, G. K. (1969), *Biochem. J. 114,* 419.
Rosen, N. L., Bishop, L., Burnett, J. B., Bishop, M. and Colman, R. F. (1973), *J. Biol. Chem. 248,* 7359-7369.
Schaeffer, H. J., and Odin, E. (1966), *J. Med. Chem. 9,* 576.
Smith, E. L., Landon, M., Piszkiewicz, D., Brattin, W. J., Langley, T. J., and Melamed, M. D. (1970), *Proc. Nat. Acad. Sci. U.S. 67,* 724.
Smith, E. L., and Piszkiewicz, D. (1973), *J. Biol. Chem. 248,* 3089-3092.

Structural Proteins of Dogfish Skeletal Muscle†

Dean A. Malencik,‡ Claus W. Heizmann,§ and Edmond H. Fischer*

ABSTRACT: As part of a study on the evolutionary aspects of control mechanisms, a number of structural muscle components from the Pacific dogfish (*Squalus acanthias*) are described. These include troponin, tropomyosin, actin, and myosin. Troponin (mol wt 108,000) was resolved into its constitutive subunits, represented by a 20,500 mol wt fragment which binds 2 mol of Ca^{2+}/mol with a K_{Diss} of 0.91 μM, and an inhibitory component of 30,000 and a 58,000 component which are necessary for the calcium sensitivity of actomyosin ATPase. Tropomyosin and actin share many properties with their counterparts from higher vertebrates. Proteins similar to parvalbumins, *i.e.*, the low molecular weight calcium-binding proteins widely distributed in fish, amphibians, and mammalian muscle, could be generated from troponin and its calcium-binding subunit by limited proteolysis. The appearance of immunological cross-reactivity and other similar features suggested some identity, but differences in the amino acid analysis exclude the possibility that parvalbumins occur as breakdown products of troponin. The close relationship between parvalbumins and the calcium-binding subunit brings additional evidence that these proteins have arisen through divergent evolution.

An investigation of the structure, function, and interrelationship of calcium-binding proteins in the muscle from the Pacific dogfish (*Squalus acanthias*) was undertaken some time ago as part of a study of the evolutionary aspects of the control of glycogen metabolism.

In this tissue, glycogen breakdown is primarily initiated by the conversion of phosphorylase *b* to *a*, catalyzed by phosphorylase kinase, an enzyme that is totally inactive in the absence of Ca^{2+} ions. Release of calcium from the sarcoplasmic reticulum or other subcellular structures triggers the activation of phosphorylase kinase and therefore, in

† From the Department of Biochemistry, University of Washington, Seattle, Washington 98195. *Received August 15, 1974.* This work was supported by grants from the National Institutes of Health (AM 07902-11), the National Science Foundation (GB 33189X), and the Muscular Dystrophy Association of America.

‡ U.S. Public Health Service Postdoctoral Fellow, GM 53362.
§ Recipient of a Swiss National Foundation Research Fellowship. Present address: ETH-Zürich, Institut für Zellbiologie, Labor für Entwicklungsbiologie, 8006 Zürich, Switzerland.

turn, that of phosphorylase. Likewise, in higher vertebrates, Ca^{2+} triggers muscle contraction by interacting with the troponin–tropomyosin complex which maintains actin and myosin dissociated in the absence of Ca^{2+}. These regulatory proteins are located in the thin filament and, therefore, act at the level of actin (Ebashi and Endo, 1968).

Another regulatory system restricted to primitive invertebrates functions directly at the myosin level (Lehman *et al.*, 1972). In both systems, interaction between actin and myosin is prevented in the absence of Ca^{2+}. Upon Ca^{2+} release the metal ion binds to a specific regulatory protein, allowing contraction to occur.

The calcium concentrations needed for muscle contraction and glycogen breakdown (≥ 1 μM) are similar since the binding constants of troponin and phosphorylase kinase for this metal ion are of the same order of magnitude (Ebashi *et al.*, 1968; Heilmeyer *et al.*, 1970; Brostrom *et al.*, 1971).

The Pacific dogfish was selected because it is readily accessible in this geographic area and it represents an early vertebrate with a well-developed endocrine system. Since it separated from the main phylogenetic line some 450 million years ago, we hoped that a study of its regulatory processes might reveal certain basic mechanisms not immediately apparent in more advanced species, assuming that the more essential features would be conserved.

This publication describes the purification and characterization of several dogfish skeletal muscle proteins; their chemical and biological properties are compared to those of analogous components obtained from higher vertebrates.

Materials and Methods

Dogfish were netted in the waters of Puget Sound and kept alive in a 4000-gallon, donut shaped tank with circulating sea water kindly provided by the Department of Fisheries, University of Washington. The fish were sacrificed as needed and the white skeletal muscle was collected for immediate processing.

Troponin was prepared according to Greaser and Gergely (1973) with slight modifications. It was first purified on a column of DEAE-cellulose equilibrated with 40 mM Tris-HCl buffer (pH 7.5), and eluted with a KCl gradient from 0 to 0.5 M in the same buffer. The isolated material was passed through DEAE-cellulose for removal of remaining impurities and degradation products (Van Eerd and Kawasaki, 1973). Troponin was resolved into its subunits by applying the lyophilized DEAE-elutant to a column of DEAE-Sephadex A-25 equilibrated with 6 M urea, 1 mM dithiothreitol, 20 mM Tris-HCl buffer, at pH 8.0 and room temperature; the subunits were eluted with a linear salt gradient from 0 to 0.6 M KCl. Urea was purified by passage over a column of mixed-bed resins (Hirs, 1967).

Tropomyosin was isolated in the course of the troponin preparation by use of an isoelectric precipitation at pH 4.6 (Hartshorne and Mueller, 1969). *Myosin* was prepared by the method of Nauss *et al.* (1969), and *actin* from an acetone powder (Spudich and Watts, 1971). *Dogfish parvalbumin* was purified according to Pechère *et al.* (1971). *Antibodies* against homogeneous parvalbumin were elicited in the rabbit.

Protein concentration was routinely measured according to Lowry *et al.* (1951). For determination of the absorbance indices the concentration of the pure proteins was determined by the synthetic boundary procedure of Babul and Stellwagen (1969). Optical measurements were made on a Cary 15 spectrophotometer and circular dichroism (CD) spectra on a Cary 60 spectropolarimeter.

Measurement of troponin activity was carried out according to Greaser and Gergely (1971) by determining the inhibition of reconstituted actomyosin ATPase in the presence of tropomyosin and EGTA.[1]

Assays were conducted at 30° in 2 ml of a reaction mixture containing 25 mM Tris-HCl, 25 mM KCl, 3.2 mg of myosin, 0.8 mg of actin, 0.4 mg of tropomyosin, 0.4 mg of troponin, 5 mM $MgCl_2$, 5 mM ATP, and either 10 mM $CaCl_2$ or 1 mM EGTA (pH 7.5). The reaction was initiated with ATP and terminated after 5 or 10 min by addition of 1 ml of 20% trichloroacetic acid. Inorganic phosphate liberated during the reaction was determined by the procedure of Fiske and Subbarow (1925). The specific activities of *reconstituted* actomyosin ATPase were in the range of 0.10–0.14 μmol of P_i per min per mg of actomyosin in the presence of EGTA or Ca^{2+} medium, respectively. Assays with *desensitized* actomyosin isolated by the procedure of Schaub and Perry (1971) and carried out in the presence of 3 mg of actomyosin, 0.5 mg of tropomyosin, and 0.5 mg of troponin gave, consistently, specific activities of 0.1 and 0.20 μmol of P_i per min per mg in the presence of EGTA or Ca^{2+}, respectively.

Polyacrylamide gel electrophoresis with or without 0.1% sodium dodecyl sulfate was performed according to Shapiro *et al.* (1967) and Weber and Osborn (1969). Unless otherwise stated, molecular weights were estimated by dodecyl sulfate gel electrophoresis. *Guanidine hydrochloride* column estimates of molecular weight were performed according to Mann and Fish (1972).

Ultracentrifuge analyses were carried out at 20° in a Spinco Model E ultracentrifuge equipped with Schlieren and Rayleigh interference optics. Sedimentation velocity experiments were carried out in 20 mM Tris-HCl–0.2 M KCl buffer (pH 7.5). High-speed sedimentation equilibrium experiments (Yphantis, 1964) were carried out in the same buffers; M_n, M_w, and M_z values were computed according to the program of Teller *et al.* (1969).

Calcium binding experiments were performed in 20 mM Tris-HCl buffer (pH 7.5) containing variable amounts of $MgCl_2$ and 0.1 M KCl according to the dynamic equilibrium dialysis procedure of Colowick and Womack (1969). Contamination by Ca^{2+} in salt and buffer solutions was always at the limit of detection and less than 0.5 μM. All solutions were stored, handled, or transferred in plastic ware. Calcium was determined in a Perkin-Elmer atomic absorption spectrometer using "Spec-pure" salts as standards. Protein bound calcium was removed by dialysis against the above buffer containing 2 mM EGTA and 2 mM Mg^{2+} for 48 hr at 4°; EGTA and Mg^{2+} were then removed by extensive dialysis against fresh buffer.

Amino acid analyses were carried out on a Beckman 120C analyzer on samples hydrolyzed in 6 N HCl for 24, 48, and 72 hr at 110° in sealed ampoules evacuated after several rinsings with pure nitrogen. Tryptophan was measured by the method of Edelhoch (1967). For determination of 1- and 3-methylhistidine, the samples were hydrolyzed 48 hr and analyzed on an Aminex Q-150 S column (20 cm) in a two-step procedure (sodium citrate, pH 4.26 and 5.28).

[1] Abbreviations used are: EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N'*-tetraacetic acid; EDTA, ethylenediaminetetraacetic acid; TN-T, tropomyosin binding subunit; TN-I, subunit that inhibits ATPase activity of actomyosin; TN-C, Ca^{2+} binding subunit of troponin; Tris, tris(hydroxymethyl)aminomethane.

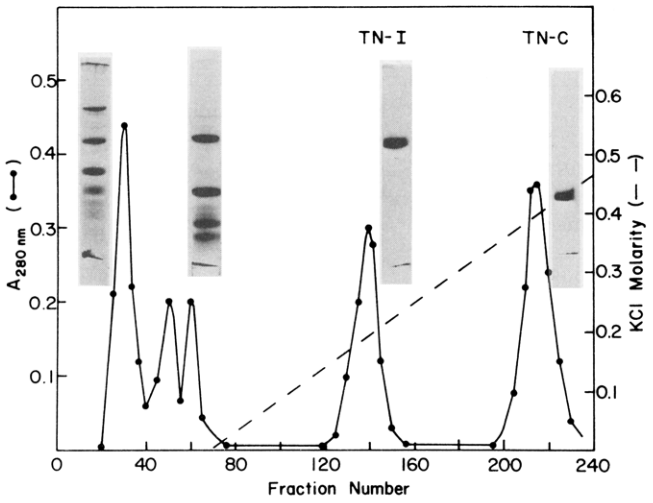

FIGURE 1: DEAE-Sephadex A-25 chromatography of troponin; 1.6 g of troponin was applied to a 50 × 4 cm column equilibrated with 6 M urea–20 mM Tris-HCl–1 mM dithiothreitol at pH 8.0. The column was washed and a linear gradient containing 0–0.6 M KCl was applied. Dodecyl sulfate gels (10%) are also shown corresponding from left to right to the original material, the breakthrough peaks (fractions 20–80), TN-I and TN-C.

Table I: Molecular Weight Data for Dogfish Troponin.

	Troponin	TN-C	TN-I	TN-T
Sedimentation velocity,[a] $s_{20,w}$	5.1 S	2.1 S ± 0.1		
Sedimentation equilibrium[a]		M_n 19,000 M_w 20,500 M_z 22,300		
Gdn·HCl column chromatography[b]		21,000 ± 1000	31,000[c]	
Dodecyl sulfate polyacrylamide gel electrophoresis	108,000 ± 7000	20,000 ± 1000	30,000 ± 2000	58,000 ± 4000

[a] 20 mM Tris-HCl–0.2 M KCl (pH 7.5). [b] Reduced, carboxymethylated with iodoacetic acid, and run on 6% agarose. [c] Largest and major protein component; other minor components with mol wt as low as 20,000 presumably resulting from proteolysis.

Results

A major difficulty encountered in the isolation of dogfish troponin was the presence of proteolytic activity that could never be entirely eliminated; evidence for this is described later. As a result, the purified troponin obtained always showed the presence of minor bands on dodecyl sulfate gel electrophoresis in addition to the three major ones corresponding to TN-T, TN-I, and TN-C. These are ascribed to degradation products rather than contamination by foreign material because they increase in the course of purification. Furthermore, TN-T undergoes spontaneous degradation to such an extent that it could never be isolated in truly homogeneous form. It is not known whether this limited proteolysis occurs at the time of death of the animal and subsequent disruption of the muscle tissue, or whether certain components of troponin are already nicked in the intact animal. Addition of usual protease inhibitors such as phenylmethanesulfonyl fluoride, diisopropyl fluorophosphate, and *N*-α-*p*-tosyl-L-lysine chloromethyl ketone did not help; attempts to minimize losses of TN-T and TN-I by applying the isolation procedures of Ebashi *et al.* (1971) and Wilkinson *et al.* (1972) were unsuccessful.

Dissociation of Troponin and Separation of Subunits are illustrated in Figure 1; TN-I and TN-C were retained on the column and emerged at 0.2 and 0.4 M KCl, respectively. The breakthrough consisted mainly of undissociated troponin and degradation products even though a relatively homogeneous preparation was applied. The largest peak attributed to TN-T was never recovered in an entirely homogeneous form. TN-I was also somewhat unstable; upon standing for 24 hr at 4°, approximately half the original material disappeared on dodecyl sulfate gel electrophoresis while small peptide fragments were produced. On the other hand TN-C was stable as long as it was saturated with Ca^{2+} ions as will be detailed later.

As in the mammalian system, dogfish troponin represents a strongly interacting system that is not completely dissociated even in the presence of 6 M urea. Attempts to separate the subunits at 4° rather than at room temperature resulted in very little dissociation. Addition of Ca^{2+} to the 6 M urea solution increased the solubility of troponin but hindered its dissociation.

Upon carrying out the DEAE-Sephadex column chromatographies at lower temperatures (*e.g.*, between 10 and 15°) different amounts of a (TN-I - TN-C) complex could be isolated. This complex was stable in the presence of Ca^{2+} and could be used in studies involving TN-I. Tropomyosin and actin were purified to homogeneity as evidenced by the appearance of single bands on gel electrophoresis (not illustrated but figure submitted to the reviewers for examination); there was no evidence of a double band for tropomyosin as reported for the rabbit protein (Cummins and Perry, 1972).

Molecular Weights of Dogfish Troponin and Its Subunits. Dogfish troponin (Table I) is larger than its rabbit counterpart (90,000 according to Greaser and Gergely, 1973). The $s_{20,w}$ value of 5.1 S is consistent with an approximately spherical molecule of mol wt 108,000.

TN-C, the Ca^{2+} binding subunit of troponin could be characterized best. All methods agreed with a mol wt of 20,500 ± 1000 (Table I), a value slightly larger than that reported for rabbit TN-C (Collins *et al.*, 1973). Sedimentation equilibrium experiments indicated the presence of a few per cent of low molecular weight material even though the protein was filtered through a Sephadex G-25 column before centrifugation.

TN-I was characterized by its ability to inhibit actomyosin ATPase and to strongly associate with TN-C. A mol wt of 30,000 ± 2000 was obtained by dodecyl sulfate polyacrylamide gel electrophoresis, and 31,000 by chromatography on a 6% agarose column run in the presence of 6 M guanidine-HCl. The instability of this material precluded the use of other physicochemical approaches.

TN-T was identified by its ability to combine with tropomyosin. It could not be characterized fully because of its unstable nature. When combined with TN-I and TN-C in the form of undissociated troponin, it cosedimented with

Table II: Amino Acid Composition of Dogfish Ca^{2+} Binding Components.[a]

Amino Acids	Number of Residues per Molecule		
	TN-C	Parvalbumin	11,000 Mol Wt Fragment
Lysine	11.4	11.8	12.7
Histidine	1.0	0.9	2.1
Arginine	5.5	1.8	4.4
Aspartic acid	25.9	15.7	11.2
Threonine[b]	8.6	5.7	5.4
Serine[b]	6.7	4.7	5.2
Glutamic acid	39.6	9.8	15.2
Proline	1.6	0.9	0
Glycine	14.1	6.9	10.3
Alanine	11.3	15.2	10.0
Valine	7.9	6.5	8.0
Methionine	9.0	1.3	2.0
Isoleucine	8.9	5.1	5.2
Leucine	12.7	12.2	6.8
Tyrosine	1.9	0.8	1.9
Phenylalanine	10.7	8.6	5.3
Cysteic acid[c]	0.8	1.0	
Tryptophan[d]	1.8	0	0

[a] Average of 24-, 48-, and 72-hr hydrolysis carried out on two preparations of TN-C, two of parvalbumin, and one of the fragment. [b] Extrapolated to zero time of hydrolysis. [c] Determined as cysteic acid according to Hirs (1967). [d] Measured spectrophotometrically according to Edelhoch (1967).

Table III: Amino Acid Composition of Muscle Components.[a]

Amino Acids	Number of Residues per Molecule		
	Tropomyosin	Actin	TN-I
Lysine	37.6	21.3	32.0
Histidine	2.2	9.1	4.3
Arginine	13.2	17.4	12.1
Aspartic acid	34.4	37.3	35.8
Threonine[b]	9.5	26.5	10.6
Serine[b]	14.3	27.7	16.5
Glutamic acid	72.5	44.8	39.8
Proline	0.2	18.6	12.7
Glycine	6.1	31.4	25.2
Alanine	36.0	32.6	29.1
Valine	11.6	21.6	19.3
Methionine	7.8	12.1	6.6
Isoleucine	10.2	29.6	13.3
Leucine	34.4	30.8	19.4
Tyrosine	6.1	16.4	3.1
Phenylalanine	2.3	12.9	6.6
Cysteic acid[c]	1.2	4.9	2.7
Tryptophan[d]	0.3	4.2	
1-Methylhistidine[e]		0	
3-Methylhistidine[e]		1.0	

[a] Average of 24-, 48-, and 72-hr hydrolysis on two separate preparations of the protein. [b] Extrapolated to zero time of hydrolysis. [c] Determined as cysteic acid according to Hirs (1967). [d] Measured spectrophotometrically according to Edelhoch (1967). [e] As described in the method of Kuehl and Adelstein (1969).

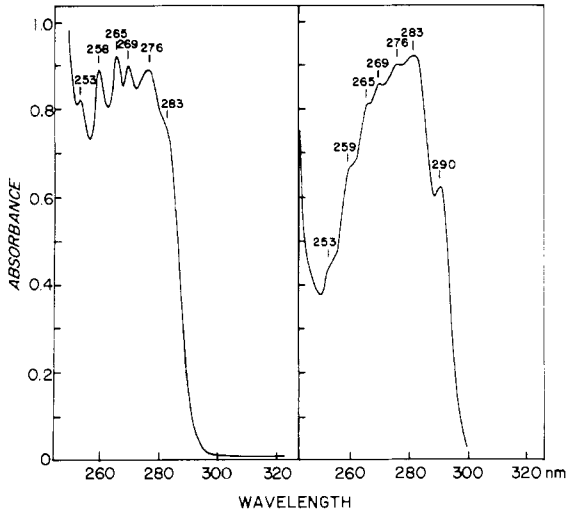

FIGURE 2: Ultraviolet spectrum of dogfish parvalbumin (left) and dogfish TN-C (right) in 2 mM Tris-HCl (pH 8.0). The protein concentrations were 5.4 and 1.3 mg/ml, respectively.

tropomyosin and F-actin upon centrifugation at 100,000*g* for 1 hr at 4°.

Amino Acid Analysis. The amino acid composition of dogfish TN-C is listed in Table II; it presents the same distinctive features as found in other TN-C molecules in that it displays low levels of histidine and proline, and a high ratio of (aspartic plus glutamic acid) to (lysine plus arginine).

The uv spectrum shows considerable vibronic structure in the region below 275 nm due to a high phenylalanine content; however, the presence of two tryptophanyl residues (in contrast to none found in rabbit and dogfish parvalbumin) alters the spectrum in the 280-nm region (Figure 2). The absorbancy index measured refractometrically in the ultracentrifuge of dogfish TN-C was approximately five times greater than rabbit TN-C and dogfish parvalbumin and gave an $A_{280}(1\%)$ of 7.0. In analogy with rabbit TN-I, dogfish TN-I has a high proline content (Table III).

Dogfish tropomyosin, the fibrous protein which lays in the groove of polymerized actin, has a subunit molecular weight estimated at 34,000 by gel electrophoresis; this value is identical with that of rabbit tropomyosin and, in fact, when the two proteins are mixed, a single band is observed. The distinctive feature of the amino acid composition of dogfish tropomyosin (Table III) is the absence of tryptophan and proline which is helpful in assessing the purity of the preparation. This amino acid composition is very similar to that of rabbit tropomyosin; likewise, CD measurements at 208 nm are consistent with a structure containing up to 90% helical regions as reported for the rabbit protein (Urnes and Doty, 1962).

Actin isolated according to the procedure of Spudich and Watts (1971) has a molecular weight estimated at 44,000 by gel electrophoresis. The amino acid composition shown in Table III is very similar to that of the rabbit protein as reported by Elzinga and Collins (1972). Rabbit actin contains 1 mol/mol of 3-methylhistidine (Asatoor and Armstrong, 1967), the same as that found in the dogfish protein.

Regulatory Function of Dogfish Troponin. Control of muscle contraction can be exercised at the actin or myosin

Table IV: Effect of Regulatory Proteins on the ATPase Activity of Dogfish Actomyosin.

Proteins	Mg-ATPase in the Presence of Ca^{2+}	MG-ATPase in the Presence of EGTA[a]
Natural actomyosin	0.20	0.08
Natural actomyosin + rabbit actin (1 mg)	0.20	0.18
Natural actomyosin + rabbit myosin (3 mg)	0.21	0.1
Desensitized actomyosin	0.18	0.18
Desensitized actomyosin + troponin (0.4 mg) + tropomyosin (0.4 mg)	0.18	0.1
Densitized rabbit actomyosin + dogfish troponin (0.4 mg) + dogfish tropomyosin (0.4 mg)	0.26	0.12

[a] Micromoles of P_i released per min per mg of protein at 30° in 25 mM Tris-HCl–25 mM KCl–5 mM $MgCl_2$–5 mM ATP (pH 7.5) in the presence of either 10 mM $CaCl_2$ or 1 mM EGTA.

Table V: Ca^{2+} Binding to Dogfish TN-C.[a]

Additions[b]	Ca^{2+}_{total} (μM)	Ca^{2+}_{free} (μM)	Moles of Ca^{2+} bound per mole of TN-C
2 mM Mg^{2+}	5	5	1.9
20 mM Mg^{2+}	5	5	1.2
2 mM EGTA	5	0	0.8
2 mM EGTA, Mg^{2+}	5	0	0.2
2 mM Mg^{2+}	50	50	2.1
Simulated physiological[c] conditions	200	50	2.2

[a] Measurements were performed by atomic absorption. Protein concentrations varied between 0.25 and 1.0 mg/ml. Free Ca^{2+} concentration was calculated from the stability constants reported by Kerrick and Donaldson (1972). [b] Samples were dialyzed against 20 mM Tris–0.1 M KCl buffer (pH 7.5) containing compounds indicated in the table, except for the last experiment. [c] Dialyzed against 3.6 mM Mg^{2+}–70 mM KCl–15 mM creatine phosphate–210 mM imidazole, 2.1 mM ATP–0.1 mM EGTA (pH 7.5).

level, or both (Lehman *et al.*, 1972). In the first instance, the addition of pure actin to an actomyosin complex activates ATP hydrolysis. On the other hand, when regulation occurs *via* the thick filament, the rate of ATP hydrolysis remains low but is increased by the addition of purified rabbit myosin under relaxing conditions.

To determine which type of regulation occurs in dogfish muscle, the method of Lehman *et al.* (1972) was followed. Dogfish troponin proved to be solely responsible for the Ca^{2+} sensitivity of dogfish muscle (Table IV), as found in all vertebrate muscle so far investigated (Weber and Murray, 1973).

Furthermore, addition of the dogfish troponin–tropomyosin system to rabbit actomyosin inhibits the ATPase in the absence of Ca^{2+} ions. This lack of species specificity is not surprising in view of the essential similarities of the regulatory proteins between the two systems. The limited calcium sensitivity afforded by troponin when added to either desensitized dogfish or rabbit actomyosin (44 and 54% inhibitor, respectively, see Table IV) might be due to the susceptibility of troponin to proteolysis.

Phosphorylation of muscle structural proteins with various phosphokinases was reported by Stull *et al.* (1972); Pratje and Heilmeyer (1972); and Perrie *et al.* (1972). The possible phosphorylation of the dogfish muscle proteins including troponin and its subunits as well as tropomyosin, actin, myosin, and parvalbumin was investigated. No phosphorylation could be observed in the presence of either dogfish phosphorylase kinase or protein kinase when added to a reaction mixture containing 25 mM glycerophosphate, 25 mM Tris-HCl, 15 mM β-mercaptoethanol, 0.05 mM $CaCl_2$, 10 mM NaF, 10 mM $Mg(OAc)_2$, 1 mM [γ-^{32}P]ATP, and 3.0 mg/ml of TN-I (pH 8.0) at 30°. On the other hand, incorporation of up to 1.4 mol of P_i/mol of TN-I was achieved when rabbit phosphorylase kinase (60 μg/ml) was added; phosphorylation was 50% completed in *ca.* 80 min. This rate was one-sixth to one-eighth that observed when an identical concentration of rabbit phosphorylase *b* (10 mg/ml) was used as substrate.

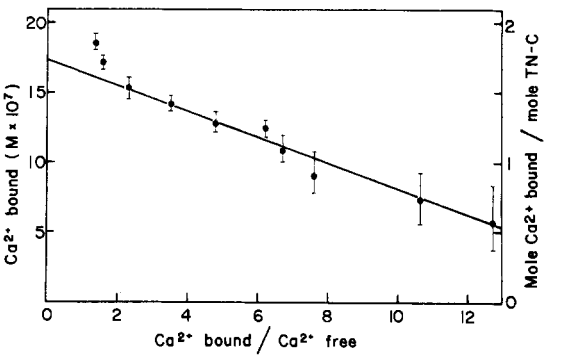

FIGURE 3: Binding of Ca^{2+} to TN-C. The *Y* axis intercept indicates 1.9 binding sites for Ca^{2+} per protein molecule of 20,500 molecular weight; the slope corresponds to a $K_{Diss.}$ of 0.91 μM.

Calcium Binding. Dogfish troponin binds 2.2 g-atoms of Ca^{2+}/mol at calcium concentrations of 50 μM, in contrast to rabbit troponin where binding of up to 4 g-atoms Ca^{2+}/mol was reported (Potter and Gergely, 1974). TN-C was the only troponin component found to possess high affinity binding sites for this metal ion. Binding studies carried out under various conditions are listed in Table V, including a physiological condition calculated by digital computer in which isometric tension of a skinned muscle fiber has been measured (Kerrick and Donaldson, 1972). It is interesting to note than one Ca^{2+} ion can be readily removed by dialysis against EGTA alone, whereas displacement of the second Ca^{2+} ion requires dialysis against Mg-EGTA.

Figure 3 shows a modifed Scatchard plot of the binding data; a K_{Diss} of 0.91 μM and a maximum $\bar{n}$ of 1.9 were determined. The upward curvature seen at high Ca^{2+} concentration is indicative of the presence of lower affinity sites ($\geq$50 μM) for Ca^{2+} on the TN-C molecule.

Relationship between TN-C and Parvalbumin. A previous communication (Heizmann *et al.*, 1974) reported that

whereas dogfish troponin does not cross-react immunologically with antiparvalbumin, breakdown products obtained by limited proteolysis (presumably slow autodigestion in the absence of Ca^{2+} ions of either purified troponin or TN-C) showed considerable cross-reactivity. Fragments of molecular weight approximately 11,000 which bind Ca^{2+} were observed in the digest.

The degradation products which emerged in the breakthrough of the DEAE-chromatography devised for the separation of the troponin subunits (see Figure 1, fractions 20–80) were further purified on a column of Sephadex G-75, yielding two major peaks. Material obtained from the first peak did not crossreact with antiparvalbumin and proved to be undegraded troponin while material from the second peak gave a strong precipitin reaction; gel electrophoresis showed the presence of two proteins with molecular weight of *ca.* 11,000 and 13,000, similar to those obtained for dogfish parvalbumins.

Final purification of the breakdown product was achieved on DE-52 column chromatography followed by elution with a 0.05–0.4 M KCl gradient. A rather homogeneous protein fragment emerging at 0.25 M KCl, with a molecular weight of approximately 11,000 on dodecyl sulfate polyacrylamide gel electrophoresis, was obtained. Its amino acid composition is shown in Table II.

A minor contaminant copurified with this fragment and migrated with the dye on gel electrophoresis, but too little was present for further investigation. Typically, from 3 g of troponin, no more than 2–3 mg of the purified, 11,000 mol wt fragment, cross-reacting with antiparvalbumin antibodies, could be obtained. The main reason for this poor yield is that, of course, this polypeptide appears transiently during the degradation process and undergoes further degradation with concomitant loss of cross-reactivity.

Neither dogfish parvalbumin nor the 11,000 mol wt fragment can replace TN-C in conferring calcium sensitivity to the actomyosin ATPase; furthermore, they could not reverse the inhibition induced by TN-I. These results are consistent with ultracentrifuge data indicating no interaction of dogfish parvalbumin with either troponin or TN-I.

Discussion

Procedures have been described for the purification of the dogfish troponin system. Isolation of a pure and stable TN-T (tropomyosin-binding component) was unsuccessful, probably because it has a tendency to aggregate in the absence of the other components of the complex and readily undergoes proteolysis. For this reason probably, the regulatory functions of troponin could not be restored by reconstituting the system with the pure subunits. While the material obtained did interact with the proteins of the thin filaments such as actin and tropomyosin, the possibility remains that other proteins might also do so; therefore further evidence is necessary to establish beyond doubt that this 58,000 component is indeed TN-T.

Several differences were observed between the dogfish troponin complex described here and that of the rabbit. The molecular weights were higher due mainly to a much larger component attributed to TN-T (58,000 *vs.* 37,000); the exact function of this component is still unclear and this considerable difference in size may reflect some altered functional characteristics as yet unrecognized. Similar results have been reported from the lobster (*Homerus americanus*) by Regenstein and Szent-Györgyi (1973). While the amino acid composition of dogfish TN-I was similar to that of the homologous rabbit protein, its specific inhibitory activity was only one-half. In this respect it appeared to be more closely related to rabbit heart TN-I (Syska *et al.*, 1974).

TN-C was the only troponin subunit possessing high affinity binding sites (two) for Ca^{2+}. In view of the different susceptibilities of the two bound metal ions to EGTA, one could speculate that one Ca^{2+} ion may be needed for the structural integrity of the protein while the other would confer calcium sensitivity to the interaction of actin and myosin. Thus, while the sites may be equivalent in terms of a polydentate chemical structure, cooperative interaction between them to yield a stable protein structure is a possibility. It has been reported that rabbit TN-C binds 4 mol of Ca^{2+}/mol of protein (Potter and Gergely, 1974).

On sodium dodecyl sulfate gel electrophoresis, *dogfish* TN-C with a molecular weight estimated at 20,000 migrated slightly behind *rabbit* TN-C (mol wt 17,800 as calculated from sequence analysis, Collins *et al.*, 1973).

Other data obtained here confirm this higher value. As reported for rabbit TN-C (McCubbin and Kay, 1973), dogfish TN-C undergoes aggregation in the ultracentrifuge in the presence of high concentrations of Ca^{2+} (10 mM) which are clearly outside the physiological range.

The degradation fragment of TN-C which cross-reacts with antiparvalbumin antibodies has an amino acid composition different from that of parvalbumin. This excludes the possibility that the small amount of cross-reacting material (0.1%) isolated from troponin could be attributed to trace contamination by parvalbumin (Heizmann *et al.*, 1974). The finding clearly indicates that while these two polypeptides must possess at least one common antigenic determinant, parvalbumins cannot originate as a breakdown product of the larger TN-C molecules. It can be reasonably assumed that the TN-C has evolved from a duplication of the parvalbumin gene as pointed out by Collins *et al.* (1973); because of homologous regions in the parvalbumin sequence, it was suggested that this molecule itself might have evolved from gene triplication (Kretsinger, 1972). In any event, it seems very likely that TN-C and parvalbumins represent a classical example of divergent evolution.

The unexpected immunological cross-reactivity between parvalbumins and subfragments of TN-C suggested that similar experiments should be carried out with the light chains of myosin. Indeed, they display similar molecular weights, amino acid compositions, and uv spectral characteristics as TN-C (Lowey and Holt, 1972) and one of them displays a strong affinity site for calcium ions (Morimoto and Harrington, 1974). Furthermore, regulation of the contractile process in invertebrates relies on the interaction of Ca^{2+} directly with the thick filaments.

Two dogfish myosin light chains could be isolated according to the procedure of Lowey and Holt (1972) with molecular weights of *ca.* 23,000 and 16,000, respectively, as estimated by dodecyl sulfate gel electrophoresis. Neither of these contained exchangable Ca^{2+}; furthermore, no stable 11,000 and 13,000 molecular weight intermediates could be generated upon limited proteolysis and no cross-reaction with antiparvalbumin antibodies could be demonstrated.

While the regulatory proteins of dogfish skeletal muscle differ somewhat from those of the rabbit, the two troponin complexes exhibit essentially the same functions and interactions. Likewise, the other structural proteins isolated, namely actin, tropomyosin, and myosin, appear to share

many features in common on the basis of these limited characterizations.

Acknowledgments

The technical assistance of Mr. R. B. Olsgaard and Ms. K. A. Peters is appreciated. We also thank Drs. S. Pocinwong and H. Blum for the dogfish parvalbumin preparation.

References

Asatoor, A. M., and Armstrong, M. D. (1967), *Biochem. Biophys. Res. Commun. 26,* 168.

Babul, J., and Stellwagen, E. (1969), *Anal. Biochem. 28,* 216.

Brostrom, C. O., Hunkeler, F. L. and Krebs, E. G. (1971), *J. Biol. Chem. 246,* 1961.

Collins, J. H., Potter, J. D., Horn, M. J., Wilshire, G., and Jackman, N. (1973), *FEBS Lett. 37,* 268.

Colowick, S. P., and Womack, F. C. (1969), *J. Biol. Chem. 244,* 774.

Cummins, P., and Perry, S. V. (1972), *Biochem. J. 128,* 106 P.

Ebashi, S., and Endo, M. (1968), *Progr. Biophys. Mol. Biol. 18,* 123.

Ebashi, S., Kodama, A., and Ebashi, F. (1968), *J. Biochem. (Tokyo) 64,* 465.

Ebashi, S., Wakabayashi, T., and Ebashi, F. (1971), *J. Biochem. (Tokyo) 69,* 441.

Edelhoch, H. (1967), *Biochemistry 6,* 1948.

Elzinga, M., and Collins, J. H. (1972), *Cold Spring Harbor Symp. Quant. Biol. 37,* 1.

Fiske, C. H., and Subbarow, Y. (1925), *J. Biol. Chem. 66,* 375.

Greaser, M., and Gergely, J. (1971), *J. Biol. Chem. 246,* 4226.

Greaser, M., and Gergely, J. (1973), *J. Biol. Chem. 248,* 2125.

Hartshorne, D. J., and Mueller, H. (1969), *Biochim. Biophys. Acta 229,* 698.

Heilmeyer, L., Meyer, F., Haschke, R. H., and Fischer, E. H. (1970), *J. Biol. Chem. 245,* 6649.

Heizmann, C. W., Malencik, D. A., and Fischer, E. H. (1974), *Biochem. Biophys. Res. Commun. 57,* 162.

Hirs, C. H. W. (1967), *Methods Enzymol. 11,* 59.

Hirs, C. H. W. (1967), *Methods Enzymol. 11,* 199.

Kerrick, W. G., and Donaldson, S. K. (1972), *Biochim. Biophys. Acta 275,* 117.

Kretsinger, R. H. (1972), *Nature (London), New Biol. 240,* 85.

Kuehl, W. M., and Adelstein, R. S. (1969), *Biochem. Biophys. Res. Commun. 37,* 59.

Lehman, W., Kendrick-Jones, J., and Szent-Gyorgyi, A. G. (1972), *Cold Spring Harbor Symp. Quant. Biol. 37,* 319.

Lowey, S., and Holt, J. C. (1972), *Cold Spring Harbor Symp. Quant. Biol. 37,* 19.

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

Mann, K. G., and Fish, W. W. (1972), *Methods Enzymol. 26,* 28.

McCubbin, W. D., and Kay, C. M. (1973), *Biochemistry 12,* 4232.

Morimoto, K., and Harrington, W. F. (1974), *Fed. Proc. Fed. Amer. Soc. Exp. Biol. 33,* 1579.

Nauss, K., Kitagawa, S., and Gergely, J. (1969), *J. Biol. Chem. 244,* 755.

Pechère, J. F., Demaille, J., and Capony, J. P. (1971), *Biochim. Biophys. Acta 236,* 391.

Perrie, W. T., Smillie, L. B., and Perry, S. V. (1972), *Biochem. J. 135,* 151.

Potter, J. D., and Gergely, J. (1974), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 33,* 1465.

Pratje, E., and Heilmeyer, L. M. G. (1972), *FEBS Lett. 27,* 89.

Regenstein, J. M., and Szent-Györgyi, A. G. (1973), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 32,* 570.

Schaub, M. C., and Perry, S. V. (1971), *Biochem. J. 123,* 367.

Shapiro, A. L., Vinuela, E., and Maizel, J. V. (1967), *Biochem. Biophys. Res. Commun. 28,* 815.

Spudich, J. A., and Watts, S. (1971), *J. Biol. Chem. 246.* 4866.

Stull, J. T., Brostrom, C. O., and Krebs, E. G. (1972), *J. Biol. Chem. 247,* 5272.

Syska, H., Perry, S. V., and Trayer, I. P. (1974), *FEBS Lett. 40,* 253.

Teller, D. C., Horbett, T. A., Richards, E. G., and Schachman, H. K. (1969), *Ann. N.Y. Acad. Sci. 164,* 66.

Urnes, P., and Doty, P. (1962), *Advan. Protein Chem. 16,* 401.

Van Eerd, J., and Kawasaki, Y. (1973), *Biochemistry 12,* 4972.

Weber, A., and Murray, J. M. (1973), *Physiol. Rev. 53,* 612.

Weber, K., and Osborn, M. (1969), *J. Biol. Chem. 244,* 4406.

Wilkinson, J. M., Perry, S. V., Cole, H. A., and Trayer, I. O. (1972), *Biochem. J. 127,* 215.

Yphantis, D. A. (1964), *Biochemistry 3,* 297.