Calcium Binding of Arterial Tropomyosin: Involvement in the Thin Filament Regulation of Smooth Muscle

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ABSTRACT: Bovine aortic tropomyosin has been isolated by DEAE-Sepharose chromatography following isoelectric precipitation and ammonium sulfate fractionation. A single polypeptide [M_r 36 000 on a sodium dodecyl sulfate (SDS)–polyacrylamide gel] was obtained under different electrophoretic conditions. The amino acid composition of bovine tropomyosin was very similar to that of rabbit skeletal muscle; the amino-terminal residue is blocked. The molecular weight of the native tropomyosin (76 000), which is twice that calculated from the SDS–polyacrylamide gel, suggests that the molecule is a dimer. The diffusion coefficient of 3.4×10^{-7} cm^2 s^{-1} and the frictional coefficient of 1.7 indicate that the molecule is asymmetric. Comparative high-pressure liquid chromatography peptide mapping of rabbit skeletal and bovine aortic tropomyosins shows primary structure variation. Bovine aortic tropomyosin binds calcium under physiological conditions of pH and ionic strength (22 mol of Ca^{2+}/mol of tropomyosin with a K_d of 1.4 mM). Such a property is not shared by skeletal tropomyosin. In low Mg^{2+} concentration, both skeletal and aortic actin activations of the skeletal myosin ATPase activity are calcium independent. Addition of aortic tropomyosin to a hybrid actomyosin (aortic actin, skeletal myosin) yields an enhancement of the actin activation of the myosin ATPase activity, but the addition of skeletal tropomyosin yields a decrease of this activity. However, both the enhancement and decrease are calcium dependent. Addition of skeletal or aortic tropomyosin to an actomyosin system, where both actin and myosin come from skeletal muscle, yields only an enhancement of the actin activation of the myosin ATPase activity. In this case, the calcium dependence of this enhancement was observed only with aortic tropomyosin. The calcium modulation obtained in the presence of aortic actin and skeletal tropomyosin suggests that the calcium site is formed by the actin–tropomyosin complex. These findings indicate that both aortic actin and tropomyosin are implicated in thin filament linked Ca^{2+} regulation of the ATPase activity of myosin in smooth muscle.

Smooth muscle contraction is triggered by an external stimulus called the first messenger. Such a stimulus, either carried by a hormone or obtained by membrane depolarization, switches on the generator–sensor–suppressor cycle of calcium, the second messenger in smooth muscle contraction (Haiech & Demaille, 1981).

As amply documented for skeletal muscles, smooth muscle contraction is mediated by the interaction of two major proteins, actin and myosin (Sparrow et al., 1970). This interaction is controlled by the calcium concentration of the sarcoplasm (Sobieszeck & Small, 1975; Adelstein & Eisenberg, 1980). This calcium modulation can potentially occur at three different levels: the polymerization and structural organization of actin, the polymerization of myosin in filamentous form through the action of a specific system including a kinase and phosphatase activity of the myosin regulatory light chain, and the actomyosin ATPase activity.

It is generally accepted that the main regulation of the smooth muscle actomyosin ATPase activity involves the phosphorylation of the 20 kilodalton light chain of myosin by myosin kinase (Hartshorne et al., 1977; Chacko & Rosenfeld, 1982) and is therefore linked to the thick filament.

However, this myosin ATPase activity can be modulated by tropomyosin (Sobieszek & Small, 1977; Hirata et al., 1977; Chacko, 1981).

Alternative or additional regulatory mechanisms linked to the thin filaments are proposed by Ebashi et al. (1977) and Marston et al. (1980). The first mechanism proposes that a calcium activating factor called leiotonin, linked to the thin filaments, regulates the actomyosin ATPase; this mechanism does not necessitate the phosphorylation of myosin. The second mechanism proposes that a calcium regulatory system linked to the thin filaments is involved in the regulation of the actomyosin ATPase.

The aim of this work was to test the ability of pure smooth muscle actin and tropomyosin to activate skeletal myosin in a calcium-sensitive manner and to compare this activation with those obtained with skeletal muscle thin filaments or hybrid thin filaments. Skeletal myosin was utilized to overcome the problem of the relationship between phosphorylation and myosin ATPase activity in smooth muscles.

Indeed, in smooth muscle, the ATPase activity is dependent on the state of phosphorylation of the myosin light chain (Chacko et al., 1977); on the contrary, MgATPase activity of skeletal muscle is independent of the level of myosin phosphorylation (Persechini & Stull, 1984).

Materials and Methods

DEAE-CL 6B Sepharose, Sephacryl S300, and MonoQ-HR columns were purchased from Pharmacia. Chymostatin and leupeptin were obtained from Sigma. [^{32}P]ATP and ^{45}Ca were from Amersham. Skeletal myosin as prepared as described by Kielley & Harrington (1966).

The catalytic subunit of cAMP-dependent protein kinase was prepared as previously described (Peters et al., 1977). Skeletal tropomyosin, prepared by the method of Hodges & Smillie (1977), was a generous gift of Dr. J. F. Rouayrenic.

Phospholipid- and Ca^{2+}-dependent protein kinase (C kinase) as prepared according to Le Peuch et al. (1984) was a generous gift of Dr. C. J. Le Peuch.

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Skeletal actin was prepared according to Pardee & Spudich (1982). Bovine aortic actin was prepared according to Cavadore et al. (1985). Briefly, aortic actin was obtained from actomyosin preparations by precipitation with 100 mM $MgCl_2$ in the presence of 10 mM ATP. The precipitate obtained by centrifugation was dehydrated with acetone and then extracted with ATP/Ca^{2+} buffer, polymerized by KCl and $MgCl_2$, depolymerized by dialysis, and purified by gel filtration on AcA 44.

Protein concentrations were determined by the method of Spector (1978) using γ-globulin as standard. The $E^{1\%}_{275nm}$ of tropomyosin was calculated by using amino acid analysis as a quantitative measure of protein concentration.

Molecular Weight and Stokes Radius Determination. Analytical gel filtration experiments were performed at 4 °C on a Sepharose S300 column (120 × 1.6 cm) equilibrated in 20 mM tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl),[1] pH 7.5, 150 mM NaCl, and 0.2 mM DTT. The absorbance at 280 nm was monitored continuously. The column was calibrated by the following molecular weight and Stokes radii (in parentheses) standards: bovine serum albumin dimer, 133 500 (43 Å); bovine serum albumin monomer, 67 000 (35 Å); ovalbumin, 45 000 (28.4 Å); chymotrypsinogen A, 25 000 (20.9 Å); ribonuclease, 13 700 (16.4 Å). The void volume and total included volume were determined with thyroglobulin and dinitrophenylalanine, respectively.

Tropomyosin solution and markers were run 3 times. The Stokes radius was determined by interpolation of the linear plot of $(-\log K_{av})^{1/2}$ vs. R_s.

Polyacrylamide gel electrophoresis was carried out in the presence of 0.1% dodecyl sulfate as described by Laemmli (1970) using a discontinuous buffer system and a polyacrylamide gradient.

Two-dimensional polyacrylamide gel electrophoresis was performed according to O'Farell (1975). The Coomassie blue stained gels were scanned with a Shimadzu C-R 3A densitometer. The carbohydrate moiety of the glycoproteins was stained according to the technique of Kapitany & Zebrowski (1973) or with cationic carbocyanine dye (Campbell et al., 1983).

Sedimentation velocity and sedimentation equilibrium experiments were performed on an MSE analytical ultracentrifuge. Migration of the boundary was followed by scanning absorption optics at 280 nm. The solvent was 0.020 M Hepes, pH 7.5, containing 0.15 M KCl and 1 mM dithiothreitol. Sedimentation velocity runs were performed at 50 000 rpm at 23 °C, and protein concentration was 0.5–1.5 mg/mL. The data were normalized to standard conditions by correcting for solvent density and viscosity. The molecular weight of native tropomyosin was determined by the equilibrium method of Yphantis (1964). Equilibrium was established by 48 h at a speed of 10 000 rpm at 12 °C and a protein concentration of 0.5 mg/mL.

Viscosity and Density Determination. Viscosity measurements were performed with an Ostwald-type viscometer at 20 °C. Solutions were filtered before measurement on 0.22-μm filters (Millipore). The density of the solutions as determined by using a digital density meter (DMA 40 PARR GRAZ, Austria).

Calcium binding parameters were determined by flow dialysis according to Colowick & Womack (1969) as previously described by Haiech et al. (1979, 1980). The tropomyosin, dissolved in 150 mM KCl and 20 mM Hepes buffer, pH 7.5 (freed for calcium by the Chelex 100 column), was chromatographed through a gel filtration column (PD 10 from Pharmacia) equilibrated with the same buffer. Determination of the number of Ca^{2+} binding sites present in the Ca^{2+}-free tropomyosin was carried out in 0.15 M KCl and 20 mM Hepes buffer, pH 7.5. Total Ca^{2+} was measured by atomic absorption spectrophotometry. Bound Ca^{2+} as measured by flow dialysis using a protein solution containing a determined amount of $^{45}CaCl_2$, by the radioactivity difference between the plateaus obtained before and after addition of unlabeled $CaCl_2$.

Amino acid analyses were performed on a Beckman Model 120 C analyzer. Cysteine was determined as cysteic acid by the method of Moore (1963) and by 5,5′-dithiobis(2-nitrobenzoic acid) titration (Ellman, 1959). Phosphoamino acids were determined according to Capony & Demaille (1983) after acid hydrolysis in 2 N HCl at 110 °C for 2 h.

Proteolytic Digestion and HPLC Peptide Mapping. Rabbit skeletal tropomyosin and bovine aortic tropomyosin (3 nmol) were dissolved in 0.1 M ammonium bicarbonate, pH 8.0, and digested with chymotrypsin (enzyme/substrate ratio of 0.08) for 10 h at 37 °C. The reaction as stopped by acidification, and the digest as injected into a 3.9 × 300 mm μBondapak phenyl column (Waters). Elution was carried out at 1.5 mL/min for 25 min using a 0–60% acetonitrile linear gradient in 0.1 M sodium phosphate buffer, pH 2.29. The eluate was monitored at 230 nm.

Assay of the ATPase Activity of Myosin. The ATPase activity of myosin was measured at 25 °C in 10 mM Hepes, pH 7.0, 60 mM KCl, 3 mM $MgCl_2$, and 0.5 mM DTT. Actin and tropomyosin were incubated together in the ATPase buffer for at least 12 h at 4 °C. Myosin was then added at 25 °C and incubated for 1 h prior to ATPase activity determination along with the desired free calcium concentration maintained by a EGTA/Ca buffer. The Ca^{2+} concentration was calculated by using an apparent CaEGTA binding constant of 7.46×10^{-7} M under experimental conditions. The reaction was initiated by addition of 2 mM ATP and terminated by 5% Cl_3CCOOH. Phosphate liberated was automatically determined as described by Terasaki & Broocker (1976). The reaction was linear with time for 15 min.

Bovine Aortic Tropomyosin Purification. Fresh aortas were freed from fat and connective tissue, ground in a meat grinder for 30 s and extracted by overhead stirring for 1 h at 4 °C with 3 volumes of 0.5 M KCl, 2 mM ATP, 5 mM $MgCl_2$, 5 mM DTT, 2 mM EGTA, and 20 mM imidazole hydrochloride, pH 7.0. Leupeptin, chymostatin (1 mg of each per liter), and soybean trypsin inhibitor (10 mg/L) were used as antiproteases in the extracting buffer. The homogenate was centrifuged for 15 min at 10000*g*, and the resultant pellet was dehydrated by repeated washes with ethanol and finally ethyl ether containing 2 mM DTT. The resulting powder was air-dried at room temperature and stored at −20 °C. Tropomyosin was extracted from the powder according to the method of Bailey (1948) with slight modifications (DEAE chromatography step as a final purification).

RESULTS

Purification of Tropomyosin. Aortic tropomyosin purified by the method of Bailey (1948) was finally purified by DEAE chromatography. This step was found necessary to remove non-protein material eluted in the second peak which did not stain when treated with Coomassie blue (Figure 1).

[1] Abbreviations: Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; SDS, sodium dodecyl sulfate; EDTA, ethylenediaminetetraacetic acid; DTT, dithiothreitol; STI, soybean trypsin inhibitor; HPLC, high-pressure liquid chromatography; Cl_3CCOOH, trichloroacetic acid; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

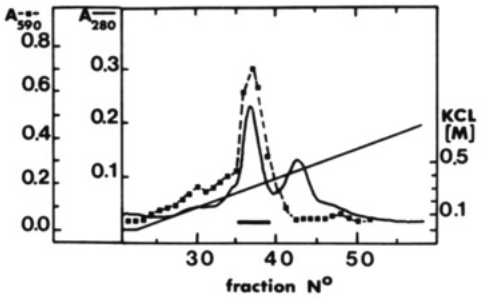

FIGURE 1: Final purification of bovine aortic tropomyosin on DEAE-Sepharose CL 6B. Following ammonium sulfate precipitation, the precipitate was dialyzed against 20 mM Tris-HCl, pH 7.5, 1 mM DTT, and 2 mM EDTA and loaded on a DEAE column (1.6 × 15 cm) previously equilibrated with the same buffer and eluted at 60 mL/h with a linear gradient of 0–1 M KCl. Fraction volume was 10 mL. The bar represents the pooled fractions. The absorbance was monitored continuously at 280 nm (solid line). Samples taken in each fraction were stained with Coomassie blue, and the absorbance was read at 595 nm (dashed line).

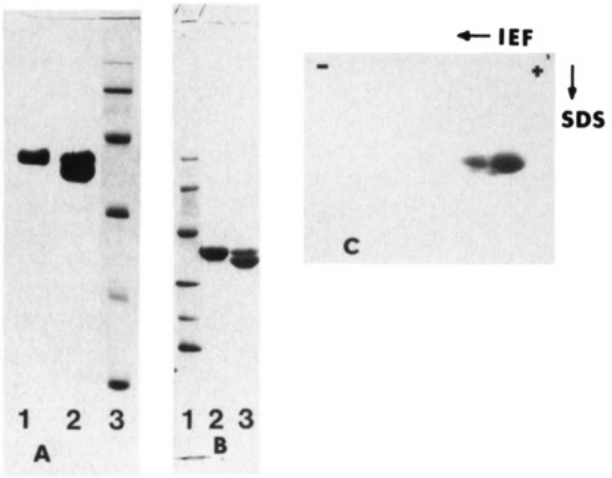

FIGURE 2: SDS–polyacrylamide and two-dimensional gel electrophoresis of bovine tropomyosin. (A) 0.1% SDS/15% polyacrylamide gel: lane 1; aortic tropomyosin (25 μg); lane 2, skeletal muscle tropomyosin (30 μg); lane 3, standard markers phosphorylase *b* (M_r 94 000), bovine serum albumin (M_r 67 000), ovalbumin (M_r 42 000), carbonic anhydrase (M_r 30 000), trypsin inhibitor (M_r 20 100), and lactalbumin (M_r 14 400). (B) 0.1% SDS/5–20% polyacrylamide gel: lane 1, standard markers identical with (A); lane 2, aortic tropomyosin (25 μg); lane 3, skeletal muscle tropomyosin (30 μg). (C) Two-dimensional electrophoresis. The isoelectric focusing was carried out at pH 4–6; the second dimension was a 15% SDS gel.

Homogeneity of bovine aortic tropomyosin was determined by gel electrophoresis under various conditions (Figure 2). After 0.1% SDS/5–20% polyacrylamide gradient gel electrophoresis and on a 0.1% SDS/15% polyacrylamide gel, aortic tropomyosin showed a single band of M_r 36 000. Two-dimensional gel electrophoresis shows the presence of two forms with a p*I* of 5.30 for the major spot and a p*I* of 5.25 for the minor spot.

Attempts to resolve the two forms by high-performance anion-exchange chromatography (FPLC monoQ Pharmacia) or hydroxyapatite chromatography were unsuccessful.

Physical Properties of Bovine Aortic Tropomyosin. Table I summarizes the physical properties of aortic tropomyosin. Comparison of the molecular weight obtained by SDS–polyacrylamide gel electrophoresis and by gel filtration indicates that native tropomyosin is a dimer. The diffusion coefficient (3.4×10^{-7} cm^2·s^{-1}) and the frictional coefficient (1.7) indicate that the molecule is asymmetric. The amino acid composition of aortic tropomyosin is very similar to that of skeletal muscle and nonmuscle cell tropomyosins (Table II). The cysteine

Table I: Physical Properties of Bovine Aortic Tropomyosin

parameter	source	value
sedimentation coefficient, $s^0_{20,w}$ (S)	analytical ultracentrifugation	3.05
diffusion coefficient, $D^0_{20,w}$ (cm^2/s)	analytical ultracentrifugation	3.4×10^{-7}
partial specific volume, $\bar{V}$ (cm^3/g)	amino acid analysis	0.724
frictional coefficient, f/f_0	s and M_r^{calcd}	1.7
mol wt, M_r	s and D	79250
	equilibrium sedimentation	76753
	gel filtration	79000 ± 700
	SDS gel electrophoresis	36000
Stokes radius, R_s (Å)	gel filtration	42.1
extinction coefficient, $E^{1\%}_{275nm}$	UV spectrum	2.16
isoelectric point	isoelectric focusing	5.3–5.25

Table II: Amino Acid Composition of Equine Platelet and Rabbit Skeletal and Bovine Aortic Tropomyosins (Moles of Residue per 36 000 g)

	rabbit skeletal tropomyosin	equine platelet tropomyosin	bovine aortic tropomyosin
Asx	29	22	27
Thr[a]	8	7.6	9
Ser[a]	15	7.2	19
Glx	70	70	70
Pro	0	0	0
Gly	3	7.2	5
Ala	36	29	34
Cys	1	2	1
Val[b]	9	10	8
Met	6	4.6	8
Ile[b]	12	9.4	6
Leu	33	29	30
Tyr	6	3.3	4
Phe	1	1.2	4
Lys	39	27	28
Arg	14	19	15
His	2	2.5	2
Trp[c]	0	0	0
total	284	255	270

[a] After extrapolation at zero-time hydrolysis. [b] From the 72-h hydrolysis value. [c] From spectroscopic evidence. Rabbit skeletal tropomyosin values are from the known amino acid sequence (Stone & Smillie, 1978), and equine platelet tropomyosin values are from Cote & Smillie (1981).

content of aortic tropomyosin is lower than that of nonmuscle tropomyosin. The only cysteinyl residue present in the molecule was accessible as determined by DTNB titration. The amino-terminal residue was found to be blocked when the molecule was submitted to Edman degradation (Edman & Begg, 1967). The comparative HPLC peptide mapping of the chymotryptic digests of skeletal and aortic tropomyosins is shown in Figure 3 and indicates substantial differences between the two types.

More than six polypeptide differences are visible which indicate important changes in the primary structure and therefore in the tertiary structure.

Unusual amino acid residues, such as methylated lysine or histidine, or sugars were not detected. The ultraviolet absorption spectrum exhibits a maximal absorbance at 275 nm and indicates the absence of tryptophan. The absorption coefficient was calculated by assuming 30 leucine and 34 alanine residues per 36 000 g and was found to be $E^{1\%,1cm}_{278nm} = 2.16$.

Phosphorylation of Tropomyosin. Attempts to phosphorylate tropomyosin with phosphorylase kinase, C kinase, and

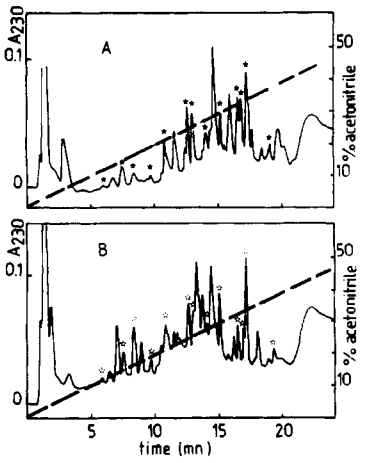

FIGURE 3: HPLC peptide mapping of the chymotryptic digest of aortic and skeletal muscle tropomyosins. Skeletal muscle tropomyosin (A) and aortic tropomyosin (B) were digested as described under Materials and Methods and separated on a μBondapak phenyl column. The dashed line represents the acetonitrile gradient. Open and closed stars mark common peptides in the two runs.

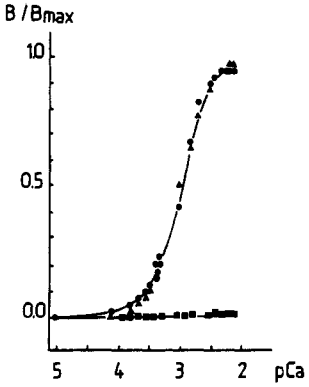

FIGURE 4: Ca^{2+} binding of skeletal tropomyosin (■) and aortic tropomyosin at 25 °C in 0.15 M KCl and 20 mM Hepes, pH 7.5 (●), and in the presence of 10 mM $MgCl_2$ (▲). The concentration was 0.22 mM for both skeletal and aortic tropomyosin. Maximal calcium bound was 4.8 mM. Bound calcium was measured by flow dialysis as described under Materials and Methods and plotted as bound/maximal bound vs. log free calcium concentration (pCa).

myosin light chain kinase were unsuccessful. Incubation with the catalytic subunit of cAMP-dependent protein kinase (enzyme/substrate ratio = 0.01, 30 min at 30 °C) resulted in very low [^{32}P]phosphate incorporation. One percent of tropomyosin molecules were phosphorylated. An increase in the enzyme/substrate ratio did not increase [^{32}P]phosphate incorporation.

Calcium Binding to Aortic Tropomyosin. When examined with the flow dialysis method (Colowick & Womack, 1969), aortic tropomyosin was shown to bind calcium (Figure 4) while identical experiments performed with rabbit skeletal tropomyosin showed no calcium binding (Figure 5). Addition of 10 mM $MgCl_2$ did not alter the calcium binding isotherm. This curve was fitted to the equation:

$$\nu = \nu_{max}\left(\frac{K_X}{1 + KX}\right)^N$$

where ν is the number of Ca^{2+} was bound per mole of tropomyosin and X is the free Ca^{2+} concentration. The dissociation constant ($1/K$) for calcium was 1.4 mM, the number of calcium binding sites (ν_{max}) amounted to 22, and N was 1.7.

Recombination Experiments of Myosin with Homogeneous or Hybrid Thin Filaments. The ATPase activity of reconstituted actomyosin was determined in the calcium concentration range 10^{-8}–10^{-5} M, with myosin/actin and tropomyosin/actin molar ratios of 1/12 and 1/4, respectively. The actin-activated ATPase of aortic acto skeletal myosin or skeletal acto skeletal myosin and their modulation by skeletal or aortic tropomyosin are reported in Figure 5.

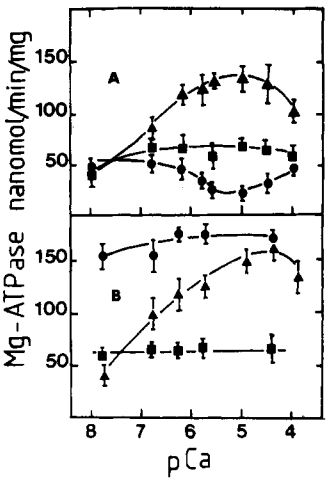

FIGURE 5: Ca^{2+} concentration dependence of the activation of skeletal muscle myosin Mg^{2+}ATPase by actin or reconstituted thin filaments. (A) Activation obtained with aortic actin (■), aortic actin + aortic tropomyosin (▲), and aortic actin + skeletal tropomyosin (●). (B) Activation obtained with skeletal actin (■), skeletal actin + aortic tropomyosin (▲), and skeletal actin + skeletal tropomyosin (●). The ATPase assay conditions were described under Materials and Methods. Points are means ±SD for six experiments.

In our conditions, both skeletal and aortic actin activation of the ATPase activity of skeletal myosin are calcium independent. When aortic actin is used, the enhancement obtained with aortic tropomyosin or the decrease obtained with skeletal tropomyosin is calcium dependent (Figure 5A). In contrast, when we substitute aortic actin with skeletal actin, both aortic and skeletal tropomyosin showed an enhancement in activity in the presence of calcium. However, only aortic tropomyosin showed a marked calcium sensitivity (Figure 5B).

In all cases, the EC_{50} of the calcium sensitivity was about 1 μM. Inhibition at Ca^{2+} concentrations higher than 10^{-4} M was observed.

Moreover, Ca^{2+} modulation was apparent when skeletal tropomyosin and aortic actin were used, indicating the possibility that the Ca^{2+} site is formed by the actin–tropomyosin complex.

Discussion

The procedure described under Materials and Methods was developed in order to yield a final purity of aortic tropomyosin greater than 97%, which permits comparison with skeletal tropomyosin. The amino acid compositions of aortic and skeletal tropomyosins do not show marked differences, except for lysine and phenylalanine. The amino-terminal residue is blocked in both skeletal and aortic tropomyosins. However, the homology between aortic and skeletal tropomyosins is not perfect, and HPLC peptide mapping of chymotryptic digests shows some marked differences, suggesting primary structure variation. These structural differences have already been reported by Fine & Blitz (1975), who, by using a fingerprint technique, showed two peptide differences between rabbit skeletal muscle and aortic smooth muscle. Our more efficient separation technique confirms and extends these observations.

The 400- and 347-Å (Fine & Blitz, 1975; Tagagi et al., 1976) periodicities of paracrystals obtained from skeletal

muscle and aorta tropomyosins, respectively, are further evidence for structural differences. Aortic tropomyosin, like skeletal and nonmuscle tropomyosin, is an asymmetric molecule. The molecular weight of native aortic tropomyosin, determined by ultracentrifugation, is twice as high as that calculated from SDS gels, suggesting that the protein is a dimer. The existence of two forms, α and β could not be shown. Indeed, the presence of phosphoserine could explain the presence of two spots after two-dimensional gel electrophoresis.

This report provides evidence for calcium binding to aortic tropomyosin under physiological conditions of pH and ionic strength. Such a property marked by its low affinity and high binding capacity is not shared by skeletal tropomyosin. This result points out a significant difference between skeletal and aortic tropomyosins. To date, these Ca^{2+} sites do not appear to play a physiological role. However, even though a simple class of binding sites is apparent in the binding isotherm, some sites may have a affinity of approximately 10^4 M^{-1}, and this affinity may be augmented in the actin–tropomyosin complex. It should also be kept in mind that tropomyosin might exhibit stronger affinities for Ca^{2+} when bound to the thin filament, as exemplified by the different affinities of native myosin and isolated myosin regulatory light chain for Ca^{2+} (Alexis & Gratzer, 1978). Skeletal tropomyosin, first assumed to be a steric blocking agent of the actin–myosin interaction (Wakabayashi et al., 1975), now is generally accepted to act as an allosteric noncompetitive inhibitor or activator by increasing or decreasing the myosin–actin interaction (Sobieszek, 1982). In smooth muscle or in nonmuscle cells, tropomyosin was shown to participate in the enhancement of ATPase activity (Sobieszek & Small, 1977; Hirata et al., 1977). The calcium sensitivity of smooth muscle contraction was linked to the phosphorylation of the 20K light chain of myosin (Hartshorne et al., 1977). The role of tropomyosin in smooth muscle was assumed to augment the actin–myosin interaction. On the other hand, smooth muscle thin filaments were shown to confer calcium sensitivity to skeletal myosin (Marston et al., 1980). We have shown here that one of the proteins implicated in this calcium sensitivity is tropomyosin.

Calcium binding to the aortic tropomyosin–actin complex appears to be important enough to modify the actomyosin ATPase activity. This finding does not rule out the possibility that other proteins participate in thin filament linked regulation. The actin and calmodulin binding protein called caldesmon (Sobue et al., 1981) also appears to be a good candidate for actomyosin ATPase modulation.

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Registry No. Ca, 7440-70-2; ATPase, 9000-83-3.

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Interaction of Troponin and Tropomyosin: Spectroscopic and Calorimetric Studies†

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ABSTRACT: The thermodynamic parameters characterizing the interaction between rabbit fast skeletal muscle troponin and tropomyosin have been determined at 25 °C for three solution conditions: buffer containing (A) 1 mM $CaCl_2$, simulating a "turned-on" state, (B) 3 mM $MgCl_2$, simulating a "turned-off" state, and (C) 2 mM ethylenediaminetetraacetic acid, a reference state. The enthalpies were measured in two buffers with different heats of ionization to allow correction for dissociation or uptake of protons. The enthalpies corrected for proton effects are −22.1, −25.4, and −23.5 kcal/mol, respectively, in buffers A, B, and C. The interaction between troponin and tropomyosin in the presence of calcium is accompanied by release of 0.9 mol of proton per mole of complex. Proton effects in the presence of magnesium and in the absence of divalent metal ions were too small to quantitate. The association constants were measured by using tropomyosin labeled with the extrinsic fluorescent probe dansylaziridine, and binding was detected by enhancement of the probe fluorescence. The magnitudes of the association constants for unlabeled troponin are 7.5×10^5, 4.2×10^5, and 9.5×10^5 M^{-1}, respectively, for the three solution conditions corresponding to unitary free energies of −10.4, −10.1, and −10.6 kcal/mol. The unitary entropies for the interaction are −39, −51, and −43 cal/(deg·mol), respectively, for the three solution conditions. Under these conditions, the troponin–tropomyosin interaction is enthalpy driven, and a large unfavorable entropy must be overcome in the formation of the complex. The troponin–tropomyosin interaction is thought to be a crucial part of the protein interactions which regulate the actomyosin ATPase activity of skeletal muscle. These studies suggest that if changes in the troponin–tropomyosin interaction are part of the regulatory signal, they are small in terms of free energy and of its enthalpic and entropic components.

The thin-filament proteins troponin and tropomyosin mediate regulation of the actomyosin ATPase of skeletal muscle by responding to the local concentration of Ca^{2+} ions. Troponin contains three subunits: TnC,[1] the Ca^{2+}-binding subunit of troponin, TnI, the inhibitory subunit of troponin; and TnT, the tropomyosin-binding subunit of troponin (Greaser & Gergely, 1971). Tropomyosin contains two parallel α-helical chains which form a coiled-coil structure that is positioned in the grooves of the thin filament [see review by Talbot & Hodges (1982)]. Troponin binds to the thin filament through interactions between TnT and tropomyosin (Mak & Smillie, 1981), TnI and actin (Potter & Gergely, 1974), and TnI and tropomyosin (Pearlstone & Smillie, 1983). Recent studies suggest that control of the actomyosin ATPase is allosteric (Chalovich & Eisenberg, 1982; Chalovich et al., 1981). Binding of Ca^{2+} to the Ca^{2+}-specific sites of TnC alters the interactions between TnC, TnI, and TnT. This in turn alters the TnI–actin and TnI– and TnT–tropomyosin interactions in a manner that strengthens the actin–myosin interaction and results in a remarkable enhancement of the actomyosin ATPase activity. Concomitant with these changes is the well-known shift of tropomyosin in the groove of F-actin (Huxley, 1971). Although little more is known of the molecular structural details, it is likely that the Ca^{2+}-induced alterations in the interactions between troponin, tropomyosin, and actin are important in the allosteric control.

An understanding of this complex system of control requires knowledge of interactions between the components. We have studied the interaction between troponin and tropomyosin by reaction calorimetry and by fluorescence spectroscopy using an extrinsic fluorescent probe under conditions where the actomyosin ATPase would be turned on and turned off. The enthalpies are of particular interest as earlier measurements

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[1] Abbreviations: TnC, Ca^{2+}-binding subunit of troponin; TnI, inhibitory subunit of troponin; TnT, tropomyosin-binding subunit of troponin; Tn, troponin; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; PIPES, 1,4-piperazinediethanesulfonic acid; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); EDTA, ethylenediaminetetraacetic acid; SDS, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane; PAGE, polyacrylamide gel electrophoresis; 1,5-IAEDANS, 5-[[[(iodoacetyl)amino]ethyl]amino]naphthalene-1-sulfonic acid; ANM, *N*-(1-anilinonaphth-4-yl)maleimide; DANZ-TM, tropomyosin labeled with dansylaziridine.

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