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Isolation and characterization of tropomyosin kinase from chicken embryo

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Tropomyosin kinase is partially purified from 14-day-old chicken embryos using DEAE-cellulose, cellulose phosphate and gel filtration chromatography. The purest enzyme preparation consists of two major bands of M_r = 76000 and 43000 on SDS-polyacrylamide gel electrophoresis. The molecular weight of the enzyme is 250000 determined by gel filtration chromatography. It phosphorylates casein and skeletal tropomyosin equally well but histone and phosvitin at a much slower rate. Smooth muscle myosin light chain, tropomyosin from platelet, erythrocyte and smooth muscle are not phosphorylated. The apparent K_m for skeletal α-tropomyosin and ATP is 50 μM and 200 μM, respectively. V_{max} varies between 100–300 nmol/min per mg depending on the purity of the preparation. Mg^{2+} and dithiothreitol are essential for activity but Ca^{+}, calmodulin and cAMP are not required. The optimum temperature is 37°C and optimum pH is about 7.5. Heparin, a potent inhibitor of casein kinase II, has no inhibitory effect on the enzyme. Similar tropomyosin kinase activity is not detected in skeletal muscle in adult rabbit and chicken. The tropomyosin kinase described here represents a hitherto uncharacterized kinase responsible for phosphorylation of tropomyosin in the chicken embryo.

Introduction

Phosphorylation of tropomyosin from skeletal and cardiac muscle in vivo and in vitro has been well documented in recent years [1–7]. Tropomyosin isolated from frog skeletal muscle previously injected with [^{32}P]orthophosphate is phosphorylated at a serine residue [1]. The in vivo phosphorylation site on rabbit skeletal muscle tropomyosin locates at serine-283, the penultimate residue [2]. While both α- and β-tropomyosin from chicken, rat and mouse skeletal muscle have been shown to be phosphorylated, the level of phosphorylation is always higher in the α subunit [6,7]. In vitro experiments have also shown that the α subunit is phosphorylated at a rate faster than that of the β subunit [3].

Although the effects of phosphorylation on the structure and function of tropomyosin are not known at present, it has been shown that the level of phosphorylation of both α- and β-tropomyosin decreases sharply during the development of the animals [4,6,7], strongly indicative of a functional role for tropomyosin phosphorylation during muscle development.

At the molecular level, it is predicted that the presence of a phosphate group at serine-283 in tropomyosin will affect the assembly and function of the tropomyosin molecule. This prediction is based on the findings that the region around

Abbreviations: EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; SDS, sodium dodecyl sulfate; PMSF, phenylmethylsulfonyl fluoride; DFP, diisopropyl fluorophosphate; Pipes, 1,4-piperazinediethanesulfonic acid.

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serine-283 is involved in the head-to-tail polymerization of the tropomyosin molecules [8,9], interaction between tropomyosin and troponin T [10,11] and the cooperative binding of tropomyosin to F-actin [12–14].

We have recently demonstrated phosphorylation of tropomyosin in vitro by a kinase from chicken embryos [3]. The in vitro and in vivo phosphorylation sites are identical, indicating that the enzyme isolated from chicken embryos is the same enzyme that phosphorylates tropomyosin in vivo. In this study, we report the partial purification and characterization of tropomyosin kinase isolated from 14-day-old thigh and leg tissue of chicken embryo.

Materials and Methods

All chemicals were of reagent grade. The following were purchased from Sigma: α-casein, histone II-A, leupeptin, pepstatin A, DFP, PMSF, bovine serum albumin and ATP. DEAE-cellulose (DE-52), cellulose phosphate (P11) and cellulose phosphate paper (P81) were obtained from Whatman. Bio-Gel A-0.5 m and A-1.5m and SDS-polyacrylamide molecular weight standards were from Bio-Rad. Fertilized chicken eggs were supplied by H and M Poultry, Harrowsmith, Ontario, Canada. [γ-^{32}P]ATP (10–12 mCi/μmol) was from Amersham.

Tropomyosin from chicken breast and leg muscle were prepared as described by Lewis and Smillie [15]. Bovine brain calmodulin was purchased from Sigma. Turkey gizzard myosin light chain was a gift from Dr. Mary Pato (University of Saskachewan).

Assay for tropomyosin kinase activity

The tropomyosin kinase fraction was added to a tropomyosin solution in 20 mM tris-HCl (pH 7.5), 10 mM $MgCl_2$, 1 mM EDTA and 0.5 mM dithiothreitol and phosphorylation was started by adding a stock solution of 10 mM [γ^{32}P]ATP (50 μCi/μmol). The final assay vol. was 25 μl and the concentration of tropomyosin and ATP was 3–5 mg/ml and 1 mM, respectively. Incubation temperature was 37°C. Covalently protein-bound phosphate was measured by the method of Glass et al. [16]. In a typical assay, 10 μl of the assay solution containing 30–50 μg of protein was spotted on a 2 cm^2 piece of Whatman P81 paper, which was washed for 4 min three times in 75 mM H_3PO_4, followed by a 1 min wash in 95% ethanol and a 1 min wash in acetone. The paper was air dried and counted for ^{32}P radioactivity in 3 ml Aquasol scintillation fluid. Background activity was determined by measuring the radioactivity of the assay solution which does not contain the substrate. Recovery of tropomyosin using this Whatman P81 paper method is determined by measuring the recovery of various amounts of ^{125}I-labelled tropomyosin treated similarly. Briefly, 1–100 μg of ^{125}I-tropomyosin was spotted on P81 papers, washed and counted as described. Identical amounts were spotted on P81 papers and counted without washing. A plot was constructed for percent recovery verses μg tropomyosin spotted on each paper and was used for correction of assays for tropomyosin phosphorylation.

Other methods

The concentration of tropomyosin was determined by measuring its absorbance at 280 nm, $\epsilon_{280\ nm}^{1mg/ml} = 0.33$ [17]. The concentration of other proteins (more than 10 μg/ml) was determined by the method of Bradford [18] using bovine serum albumin as standards. SDS-polyacrylamide gel electrophoresis was carried out as described by Laemmli [19].

To analyze dilute enzyme solution (concentrations below 10 μg/ml) on SDS-polyacrylamide gel electrophoresis, 100 μl aliquots of the samples were desalted on Millipore filter (vs. 0.02 μm), as described by Marusyk and Sergeant [20] followed by freeze drying. The dried sample was dissolved in 10 μl of 100 mM Tris-HCl (pH 6.8), 2% SDS, 10% sucrose, 1 mM dithiothreitol and heated at 100°C from 5 min, 0.5–1 μg of sample was analyzed on a 12% acrylamide SDS gel as described in Ref. 19. To determine the protein concentration of such dilute solutions, protein was precipitated with 10% trichloroacetic acid on ice for 1 h, centrifuged for 10 min at 4000 × g. Pellets were solubilized in 0.1 M NaOH and the protein contents were determined by the method of Lowry et al. [21] with bovine serum albumin as a standard.

Results

Purification of tropomyosin from 14-day-old chicken embryo

Extraction of tropomyosin kinase. Fertilized eggs were incubated for 14 days in an egg incubator (Humidaire Incubator, New Madison, OH, U.S.A.) at 37.2° C. The average embryo weighed 13.0 g. About 200 g of thigh and leg tissue was obtained from 20 dozen chicken embryos and were used for a single tropomyosin kinase preparation. The tissue was frozen immediately in liquid N_2 and used within 2 h or stored at −70° C and used within 7 days. The leg and thigh tissue was washed twice in phosphate-buffered saline to remove yolk proteins. The washed tissue was homogenized in 600 ml of 20 mM Tris-HCl (pH 7.5) 0.2 M KCl, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 2 μM pepstatin, 2 μM leupeptin and 0.5 mM DFP (which was added to the extraction mixture in a fumehood just prior to homogenization) in a Sorvall Omni-Mixer at maximum speed, four times for 30 s. each time. The homogenate was stirred for 30 min and sedimented at 26000 × *g* for 1 h. The slightly reddish supernatant was filtered through glass wool and the pellet was discarded. Solid $(NH_4)_2SO_4$ was added gradually to the supernatant over 45 min with constant stirring to give 30% saturation. The suspension was sedimented at 26000 × *g* for 30 min. The supernatant was discarded and the pellet was suspended in 50 ml of buffer A (20 mM Tris-HCl (pH 7.5), 20 mM KCl, 1 mM EDTA and 0.5 mM dithiothreitol with 2 μM pepstatin, 2 μM leupeptin and 0.5 mM DFP) and was homogenized in a teflon/glass homogenizer for 5 strokes. The fine suspension was stirred for 30 min and dialyzed against 2 l of buffer A with four changes of buffer for a total of 8 h. The dialyzed suspension was sedimented at 200000 × *g* for 1 h to remove insoluble materials. The lipid material at the surface of the supernatant was removed and the slightly cloudy supernatant with a protein concentration of approx. 3.5 mg/ml was applied to a DEAE-cellulose column.

DEAE-cellulose chromatography. The 0–30% $(NH_4)_2SO_4$ fraction is applied to a DEAE-cellulose column, 1.6 × 20 cm, which has been equilibrated with buffer A. A linear gradient of 300 ml, 20–450 mM KCl in buffer A was used to elute the tropomyosin kinase activity which elutes as a single peak at a conductance of 6–10 mmho (Fig. 1). The pooled tropomyosin kinase fraction, approx. 50 ml, is dialyzed against 2 l of buffer A with two changes of buffer for 8 h. The protein concentration was approx. 1 mg/ml.

Cellulose phosphate chromatography. The dialyzed DEAE-cellulose fraction was loaded onto a column of cellulose phosphate (Whatman P11) 1.6 × 10 cm and eluted with a linear gradient of 180 ml, 20–450 mM KCl in buffer A (Fig. 2). A major peak of tropomyosin activity eluted at a conductance of 4–6 mmho and a minor one at 8–9 mmho. The relative yield of the major and minor peak is normally 9 : 1. The minor peak is

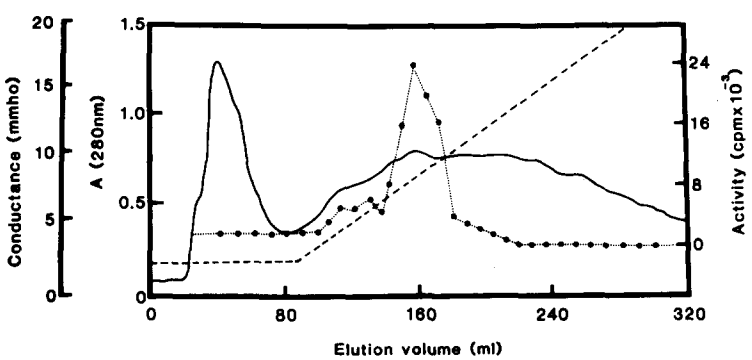

Fig. 1. DEAE-cellulose chromatography of the 0–30% $(NH_4)_2SO_4$ fraction of tropomyosin kinase. 60 ml of the 0–30% $(NH_4)_2SO_4$ fraction was applied to the DEAE-cellulose column, 1.6×20 cm, equilibrated with 20 mM Tris-HCl (pH 7.5), 20 mM KCl, 1 mM EDTA and 0.5 mM dithiothreitol. The enzyme was eluted with a linear gradient of 300 ml, 20–450 mM KCl in the equilibration buffer. The flow rate was 25 ml/h and the fraction size was 5 ml/tube. The fractions were assayed for protein concentration at A_{280} nm (———). Tropomyosin kinase activity (●———●) and conductivity (- - - - -).

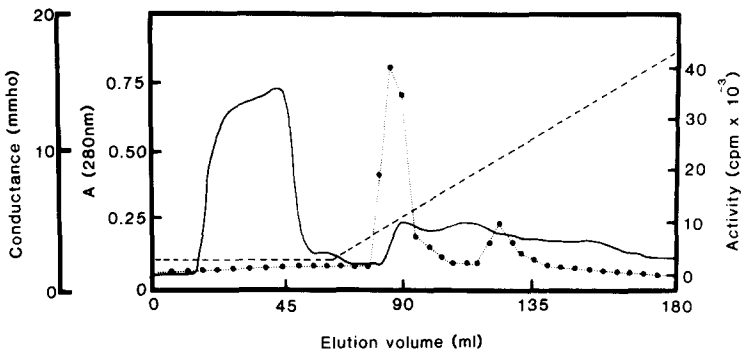

Fig. 2. Cellulose phosphate chromatography of the pooled tropomyosin kinase peak from the DEAE-cellulose column. 50 ml of the pooled DEAE-cellulose were applied to the Whatman P11 column, 1.6×10 cm, equilibrated with 20 mM Tris-HCl (pH 7.5), 20 mM KCl, 1 mM EDTA and 0.5 mM dithiothreitol. A gradient of 180 ml, 20–450 mM KCl in the equilibration buffer was used to elute the enzyme. The flow rate was 20 ml/h and 2.5 ml/tube was collected. The fractions were assayed for protein concentration (———), tropomyosin kinase activity (●———●) and conductivity (- - - - -).

probably a degraded product of the major one, since exclusion of proteinase inhibitors in the extraction buffer or extensive dialysis of the 0–30% $(NH_4)_2SO_4$ fraction against buffer A decreased the ratio of the major and minor peak. After completion of the gradient, the column was washed with 0.8M KCl in buffer A but no activity was detected in the eluant. PMSF was added to the major peak to make 50 μg/ml approx. 15 ml, and was dialyzed against 2 l of buffer A for 8 h with one change of buffer, and concentrated on a 0.5 ml DEAE-cellulose column by eluting the tropomyosin kinase with 250 mM KCl in buffer A. Solid sucrose was dissolved in the concentrated sample to give a final concentration of 10%. The final vol. was 2 ml and the protein concentration was approx. 0.6 mg/ml. Recovery of tropomyosin kinase activity was more than 90% using this method of concentrating. Other methods of concentration by ultrafiltration and dialysis against solid sucrose or poly(ethylene glycol) resulted in a much lower yield of the enzyme.

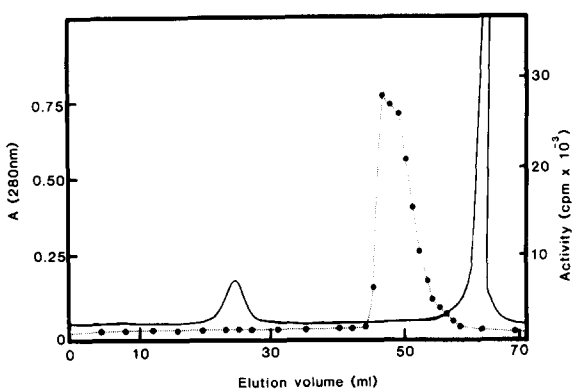

Fig. 3. Gel filtration of tropomyosin kinase. The pooled cellulose-phosphate peak of tropomyosin kinase (15 ml) was concentrated to 2 ml in a 0.5 ml DEAE-cellulose column. The concentrated sample was applied to a Bio-Gel A-1.5m column, 1×100 cm, preequilibrated with 20 mM potassium phosphate (pH 7.2), 1 mM dithiothreitol, 1 mM EDTA and 50 μg/ml PMSF. The flow rate was 3 ml/h and the fraction size was 1 ml/tube. The fractions were assayed for protein concentration (———) and tropomyosin activity (●———●).

Gel filtration chromatography. The concentrated tropomyosin kinase from the cellulose phosphate column was applied to a Bio-Gel A-1.5m column, 1.0 × 100 cm, equilibrated with buffer B (20 mM potassium phosphate (pH 7.2), 1 mM dithiothreitol, 1 mM EDTA and 50 μg/ml PMSF) (Fig. 3). A single peak of tropomyosin kinase activity eluted at $K_{av} = 0.56$. Increasing the salt content of the buffer to 150 mM KCl did not change the elution position of the enzyme peak. The pooled fraction was approx. 4 ml and had a protein concentration of approx. 2 μg/ml. It is worthwhile noting that the minor peak from the cellulose phosphate column eluted at the void volume from the Bio-Gel A-1.5m column under the same conditions (not shown).

TABLE I

SUMMARY OF PURIFICATION OF TROPOMYOSIN KINASE FROM 200 G OF LEG AND THIGH TISSUE FROM 14-DAY-OLD CHICKEN EMBRYOS

	Protein concentration (mg/ml)	Total Protein (mg)	Spec. Act. (nmol/min per mg)	Purification (fold)	Yield (%)
0.2 M KCl extract	6.6	3690	–	–	–
0–30% $(NH_4)_2SO_4$	3.7	193	0.027	1.0	100
DEAE-cellulose	1.2	43	0.087	3.2	71
Cellulose phosphate	0.068	1.0	0.620	23.0	12
Bio-Gel A-1.5m	0.002	0.010	59.4	2200	11

Comments on the purification procedures

A summary of the purification of the tropomyosin kinase is shown in Table I. The gel filtration chromatography step was most effective in the purification of the tropomyosin kinase offering a 100-fold purification with little loss in yield. Over 90% of the contaminating proteins eluted at the void volume.

The purification procedure is mostly reproducible up to the Bio-Gel A-1.5m step. At this stage, the yield of the enzyme, is usually about 10 μg from 200 g of embryonic tissue. Inclusion of soybean trypsin inhibitor or bovine serum albumin in the elution buffer as carriers does not improve the yield of the enzyme. The spec. act. of the enzyme varies from 60–300 nmol PO_4/min per mg, depending on the purity of the preparation. Further purification results in an over 80% loss of the enzyme, irrespective of the type of chromatography employed, including hydroxyapatite, phenyl Sepharose, amino hexyl and Matrex gel red A agarose chromatography. An assay of tropomyosin kinase activity after this stage is unreliable due to the minute amount (less than 2 μg) of enzyme recovered. We have made several attempts to scale up the preparation using 30–40 dozen chicken embryos, but so far we have not been successful in improving appreciably the yield of the tropomyosin kinase.

The enzyme from the cellulose phosphate column is stable for at least 2 weeks when stored in 10% sucrose at 4°C. The Bio-Gel fraction, after being concentrated to 10 μg/ml on a 0.5 ml DE-52 column, however, becomes much less stable when stored under similar conditions with 50% loss in activity in about 48–72 h.

Characterization of tropomyosin kinase

SDS-gel electrophoresis and subunit composition of tropomyosin kinase. The SDS-gel electrophoresis pattern of tropomyosin kinase after the Bio-Gel A-1.5m step shows two major bands of $M_r = 76000$ and 43000 (Fig. 4A). A minor band of $M_r = 40000$ is also visible. Since the enzyme concentration at the Bio-Gel stage is too dilute, approx. 10 μg/ml even after it has been concentrated on a 0.5 ml DE-52 column, to be analyzed using gel electrophoresis under non-denaturating conditions, the subunit composition of the enzyme is uncertain at this stage. An alternative purification scheme was therefore used to prepare the tropomyosin kinase to near homogeneity and the SDS-gel electrophoresis patterns of the two enzyme preparations were compared to determine its subunit composition.

The second purification scheme was essentially the same as the first one, except that a hydroxyapaptide column, 1.6 × 10 cm, was used instead of the cellulose phosphate column. The hydroxyapaptide column was pre-equilibrated with 20 mM KPO_4 (pH 7.2), 1 mM dithiothreitol and 1 mM EDTA and the enzyme eluted as a single peak between 200–300 mM PO_4 as shown in Fig. 5. The pooled kinase fractions were then chromatographed on a Bio-Gel A-1.5m column as described in the first purification scheme. The tropomyosin kinase eluted at the same position, $K_{av} = 0.56$, as observed before (Fig. 3). The yield of the kinase was approx. 5 μg with a spec. act. of 150 nmol/min per mg. The SDS-gel pattern of the enzyme at this stage showed a major band of $M_r = 76000$ which constituted approx. 70% by weight of all the protein bands (Fig. 4B). Although a few faintly stained

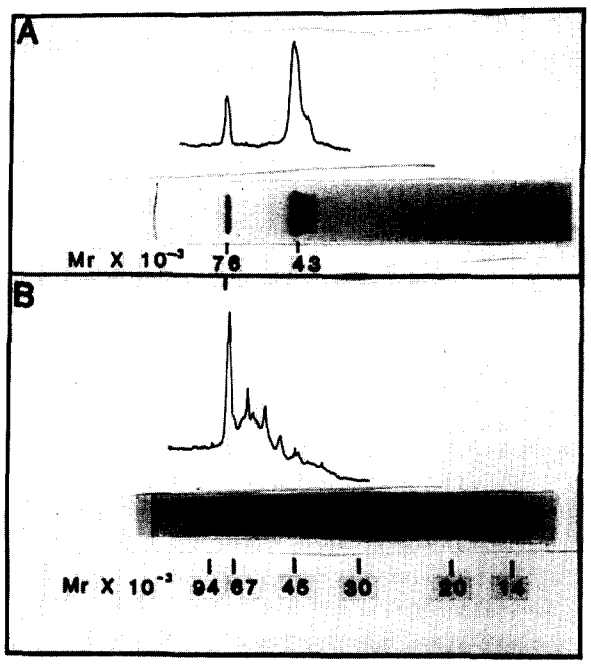

Fig. 4. SDS-gel electrophoresis of tropomyosin kinase. Approx. 0.5 μg of protein was applied in each lane. The SDS gel was 12% acrylamide. Protein standards were phosphorylase *B*, M_r 93000; bovine serum albumin, M_r 66000; ovalbumin, M_r 45000; carbonic anhydrase, M_r 31000 and soybean trypsin inhibitor M_r 14000. A densidometric scan of the Coomassie blue stained protein bands is shown on top of each gel. (A) Tropomyosin kinase obtained from purification scheme No. 1, 0–30% $(NH_4)_2SO_4$ → DEAE-cellulose → cellulose-phosphate → Bio-Gel A-1.5m. (B) Tropomyosin kinase from purification scheme No. 2, 0–30% $(NH_4)_2SO$ → DEAE-cellulose → hydroxyapaptite → Bio-Gel A-1.5m.

bands were also visible, the M_r 43000 and 40000 bands were absent. Since only the M_r 76000 band appeared in both preparations of the enzyme (Fig. 4A and 4B) it suggested that the tropomyosin kinase consists of only one subunit. Alternatively, the M_r 76000 and the 43000 bands may represent the catalytic and regulatory subunits of the tropomyosin enzyme, respectively, and become separated on the hydroxyapaptide column during the second purification procedure.

Molecular weight determination by gel filtration chromatography. Molecular weight of the tropomyosin kinase is determined by gel filtration on a Bio-Gel A-0.5m column, 0.9 × 100 cm, equilibrated with buffer A (Fig. 6). The column is precalibrated with molecular weight standards, thyroglobulin, M_r 670000; γIgG, M_r 158000;

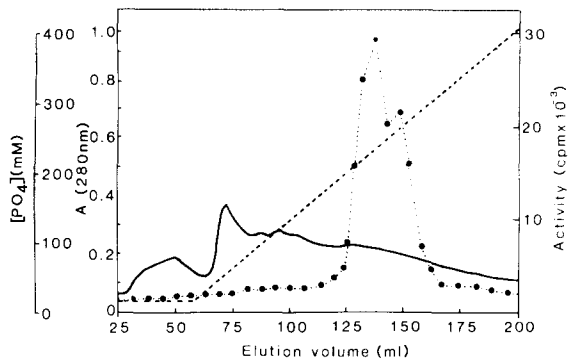

Fig. 5. Hydroxyapaptite chromatography of tropomyosin kinase (purification scheme No. 2). The pooled DEAE-cellulose peak,, 50 ml, was applied to a hydroxyapaptide column, 1.6×10 cm, preequilibrated with 20 mM potassium phosphate (pH 7.2), 1 mM dithiothreitol and 1 mM EDTA. The enzyme was eluted with a 180 ml gradient of 20–500 mM phosphate. The flow rate was 20 ml/h and fractions of 2.5 ml/tube were collected. The fractions were assayed for protein concentration (———), tropomyosin kinase (●———●) and phosphate concentration (- - - - -).

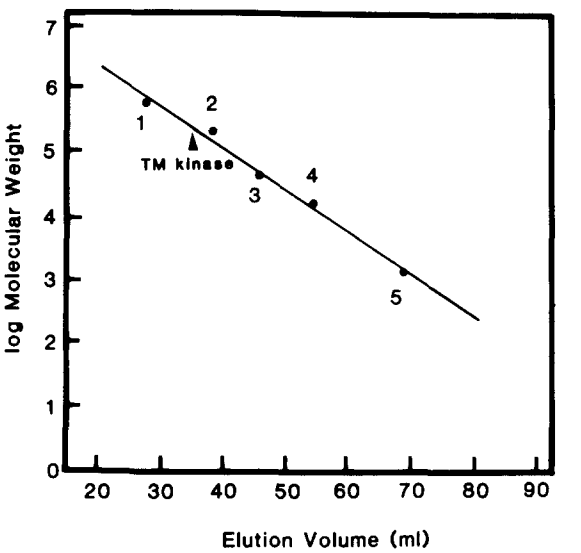

Fig. 6. Molecular weight determination of tropomyosin kinase by gel filtration. 1 ml of the tropomyosin kinase, 50 μg/ml, was applied to a Bio-Gel A-0.5m column, 0.9×100 cm, equilibrated with 20 mM Tris-HCl (pH 7.5), 20 mM KCl, 1 mM EDTA and 1 mM dithiothreitol and was eluted with the same buffer at a rate of 3 ml/h. 1 ml fractions were collected and assayed for tropomyosin kinase activity as described in the Materials and Methods section. The column was calibrated with molecular weight standards: 1, thyroglobulin (M_r 670000); 2, γIgG (M_r 158000); 3, ovalbumin, (M_r 44000); myoglobulin (M_r 17000) and vitamin B-12 (M_r 13500). The position of the peak tube of tropomyosin kinase activity is represented by the arrow.

ovalbumin, M_r 44000; myoglobin, M_r 17000; and vitamin B-12 M_r 13500.

The tropomyosin kinase elutes as a single peak before γIgG at a position corresponding to a M_r of 252000 ± 24000, obtained from four enzyme preparations. The elution position is not altered when 0.15 M KCl is included in buffer A.

Substrate specificity. The rates at which the tropomyosin kinase phosphorylates some potential substrates are shown in Table II. Casein and α-tropomyosin phosphorylated at similar rates, whereas troponin complex from rabbit skeletal muscle, phosvitin and histone II-A phosphorylated at 28%, 25% and 19%, respectively, of the rate for tropomyosin. The myosin light chain from turkey gizzard was not phosphorylated.

Tropomyosin from turkey smooth muscle, horse platelet and human erythrocyte were not phosphorylated. β-Tropomyosin purified from rabbit skeletal muscle was phosphorylated at a slower rate.

TABLE II

SUBSTRATE SPECIFICITY OF TROPOMYOSIN KINASE

The substrate was incubated with tropomyosin kinase (1 μg/ml) at 37 °C. The reaction was started by addition of [γ-^{32}P]ATP. Two 10 μl aliquots of the reaction mixture were spotted on Whatman P81 paper at appropriate time periods and protein-bound phosphate was determined as described in Materials and Methods. Maximal activity was 120 nmol/min per mg.

Substrate	Concentration (mg/ml)	Maximal activity (%)
α-Tropomyosin (skeletal muscle)	5	100
Casein	5	120
Troponin mixture (skeletal muscle)	1	28
Histone II-A	5	19
Phosphorylase *b*	1	9
Phosvitin	5	25
Myosin light chain (turkey gizzard)	1	0
β-Tropomyosin (skeletal muscle)	5	10
Non-muscle tropomyosin		
horse platelet	5	0
human erythrocyte	5	0

Kinetic properties. The tropomyosin kinase partially purified on the Bio-Gel A-1.5m column according to the first purification scheme was used for all kinetic studies. The average apparent K_m for α-tropomyosin from chicken breast muscle determined three times using different preparations of kinases was 50 μM. The extrapolated V_{max} value from different Lineweaver-Burk plots using different preparations of enzyme varied between 100–300 nmol PO_4/min per mg (Fig. 7).

In the range of ATP concentration tested, 0–1

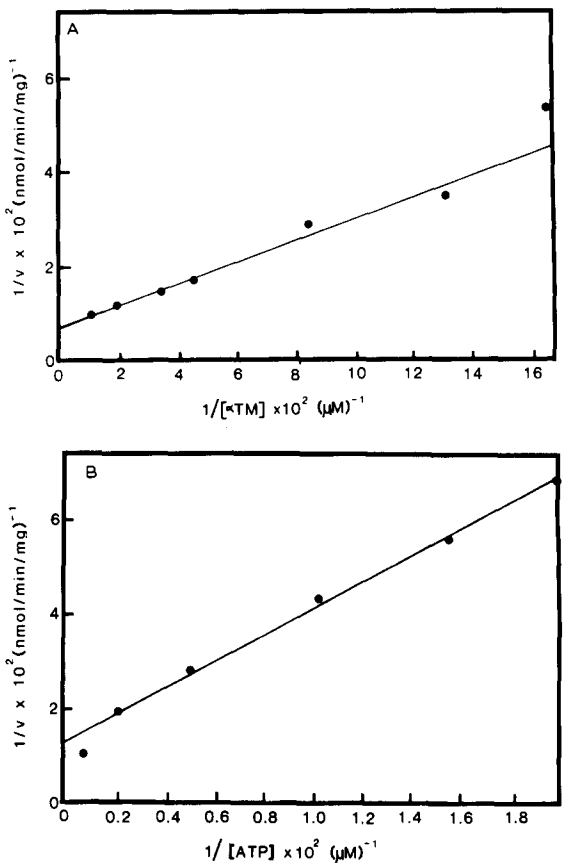

Fig. 7. Lineweaver-Burk plots of the phosphorylation of skeletal muscle α-tropomyosin by tropomyosin kinase. Phosphorylation rates were determined by measuring the amount of PO_4 incorporated in tropomyosin at the appropriate time intervals of up to 2 h. The assay procedure is described in the Materials and Methods sections. The kinetic parameters were analyzed using a non-linear regression computer program described in Ref. 33. The tropomyosin kinase concentration was 2 μg/ml. (A) The concentration of α-tropomyosin was varied from 6 to 100 μM based on subunit M_r 33000 and ATP was 1 mM. (B) The concentration of ATP varied from 50 μM to 1 mM and α-tropomyosin was kept constant at 100 μM.

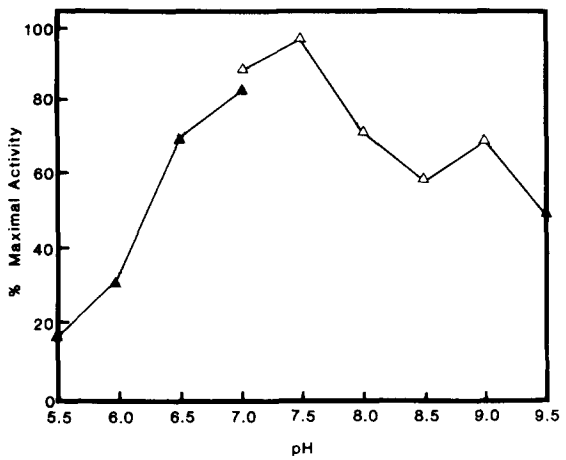

Fig. 8. Effect of pH on tropomyosin kinase activity. The tropomyosin, ATP and tropomyosin kinase concentration were 5 mg/ml, 1 mM and 2 μg/ml, respectively. Assay conditions are described in the Materials and Methods section. Maximal activity was 100 nmol/min per mg. 20 mM Tris-HCl was used as a high-pH buffer (△———△); 20 mM Pipes was used as a low-pH buffer (▲———▲).

mM, phosphorylation of α-tropomyosin appeared to follow Michaelis-Menten kinetics with an apparent K_m of 200 μM as shown in fig. 7.

Effect of pH on tropomyosin kinase activity. The activity of the tropomyosin kinase spans over a wide pH range, from pH 6.5 to pH 9.5, as shown in Fig. 8, with an optimum pH at 7.5.

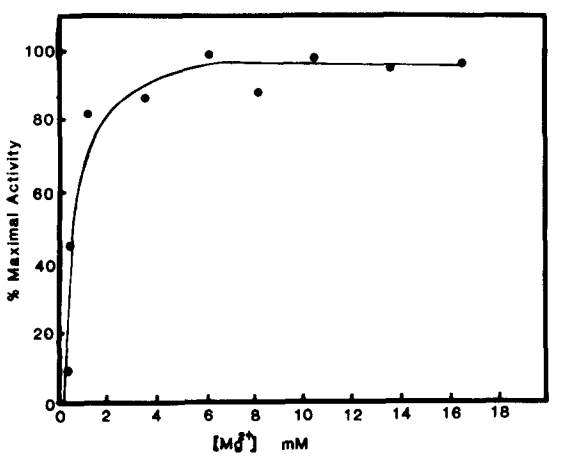

Fig. 9. Effect of Mg^{2+} on tropomyosin kinase activity. The assay conditions are the same as those described in Fig. 8. The concentration of $MgCl_2$ are expressed as free Mg^{++}. Maximal activity was 100 μmol/min per mg.

Temperature dependence of the tropomyosin kinase activity. The rate of phosphorylation of α-tropomyosin was measured at 22°C, 32°C, 37°C and 45°C (not shown). The optimum temperature was 37°C. At 22°C, the enzyme activity was only 10% that at 37°C, whereas at 45°C, 61% of the maximum activity remained.

Effects of some other factors on the tropomyosin kinase activity. As reported previously [3], tropomyosin kinase requires Mg^{2+} for activity. As shown in Fig. 9, in the absence of Mg^{2+}, no activity was detected. Activity rises with an increase in the Mg^{2+} concentration reaching a maximum at 4–5 mM Mg^{2+}.

Calmodulin in the presence or absence of Ca^{2+} does not affect the activity of tropomyosin kinase as shown in Table III.

It is known that heparin specifically inhibits casein kinase II with an apparent K_I of 20 ng/ml [27]. As shown in Table III, heparin does not inhibit tropomyosin kinase using either casein or tropomyosin as substrates.

Tropomyosin kinase displays a requirement for reducing conditions for activity as shown in Table III. No activity was detected in the absence of dithiothreitol. A similar dithiothreitol dependence was observed when casein was used as a substrate.

TABLE III

EFFECTS OF SOME FACTORS ON TROPOMYOSIN KINASE ACTIVITY

The basic reaction mixture contained 5 mg/ml α-tropomyosin, 10 mM $Mg Cl_2$, 20 mM Tris-HCl (pH 7.5), 20 mM KCl, 1 mM ATP, 0.5 mM dithiothreitol and 1 μg/ml of tropomyosin kinase incubated at 37°C. Different factors were added to the basic mixture at the concentration indicated. Two 10 μl aliquots were spotted on Whatman P81 paper at the appropriate time periods and assayed for protein bound PO_4 as described in Materials and Methods.

Factors	Maximal activity (%)
Basic reaction mixtures	100
Calmodulin (1 mg/ml) + Ca^{2+} (1 mM)	85
Calmodulin (1 mg/ml) + 1 mM EDTA	90
No dithiothreitol [a]	0
Heparin (20 ng/ml)	100
Heparin (200 ng/ml)	100

[a] Dithiothreitol was omitted from basic reaction mixture.

Discussion

Due to the limited yield and instability of the highly purified tropomyosin kinase after three columns, further purification proved futile, resulting in great loss of activity. Each of the two purification schemes alone does not purify the enzyme to homogeneity. However, SDS-gel electrophoresis of enzyme preparations obtained from the two different purification schemes showed clearly that the only major band that appears in both preparations has a M_r of 76000 (Fig. 4). This suggests that the M_r 76000 protein represents the catalytic subunit of the enzyme. However it is not clear whether the M_r 43000 band represents a regulatory subunit which is separated from the catalytic subunit on the hydroxyapaptite column in the second purification scheme. Assuming that the tropomyosin kinase is a globular protein, its molecular weight is 250000, determined by gel filtration chromatography.

We have used other purification procedures employing various column chromatographies including aminohexyl, phenyl Sepharose, Matrex gel red A agarose, ATP-Sepharose, tropomyosin-Sepharose and casein-Sepharose. All gave less satisfactory results, in yield or purification, than the two purification schemes described here. The tropomyosin kinase failed to bind to the affinity columns using ATP, tropomyosin and casein as ligands, possibly due to the high K_m values for these substrates which exceed the ligand concentration of the affinity column.

We did not detect similar tropomyosin kinase activity in adult chicken leg muscle and adult rabbit skeletal muscle. No tropomyosin kinase activity was detected in the 0–30% and 30–50% $(NH_4)_2SO_4$ salt fractions. No activity was detected after either fraction was passed through a DEAE-cellulose column although casein kinase activity was detected as reported in Ref. [24]. It appears that the level of tropomyosin kinase in the adult tissue is reduced significantly which may account for the reduced amount of phosphorylated tropomyosin in adult animals.

The tropomyosin kinase described here phosphorylated α-tropomyosin and casein equally well but histone and phosvitin served as much inferior substrates. Its activity is independent of cAMP [3], Ca^{2+} and calmodulin. Although some properties of tropomyosin kinase are similar to casein kinases [22], it is quite different from the well-characterized casein kinases I and II isolated from human erythrocyte [23], skeletal muscle [24], reticulocyte [25] and liver [26]. Casein kinase I and II bind cellulose phosphate tightly and elute at 0.5–0.7 M NaCl, tropomyosin kinase however, binds only weakly to cellulose phosphate and dissociates from it readily in 50 mM KCl. Tropomyosin kinase does not form high M_r aggregates in buffers of low ionic strength, which is in contrast to the behavior of casein kinase II [22]. Heparin, a potent and specific inhibitor of casein kinase II, does not inhibit the activity of tropomyosin kinase, again suggesting that tropomyosin kinase is not a casein kinase II-type of enzyme. Furthermore, the sequence of the phosphorylation site in tropomyosin bears little resemblance to that of other known phosphoproteins [3].

The amino acid sequence of the COOH-terminal end of the tropomyosin molecule, where the phosphorylation site is located, is highly variable among different tropomyosin isoforms expressed in different tissues, presumably a result of alternative mRNA splicing [28]. Thus, the phosphorylation site in skeletal and cardiac muscle tropomyosin is not conserved in tropomyosin from non-muscle and smooth muscle [3,29,30] and accounts for the finding that only skeletal and cardiac muscle tropomyosin can be phosphorylated by the tropomyosin kinase.

Although the phosphorylation sites in α- and β-tropomyosin are fairly homologous [3,31], α-tropomyosin is phosphorylated at a faster rate than the β isoform [3]. Based on X-ray crystallography data, it has been suggested that the head-to-tail overlap region in tropomyosin may assume a globular conformation [32] and, therefore, have a more flexible structure. Subtle differences in the conformation of the α- and β-tropomyosin at the head-to-tail region may account for the observed difference in the phosphorylation rates.

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