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PURIFICATION AND PROPERTIES OF TROPONIN T KINASE FROM RABBIT SKELETAL MUSCLE

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Summary

A protein kinase (ATP:protein phosphotransferase, EC 2.7.1.37) which catalyzes the phosphorylation of troponin T, phosvitin and casein has been purified over 2000 fold from rabbit skeletal muscle. The partial purification of this new enzyme, designated troponin T kinase, involves precipitation of contaminating proteins at pH 6.1, fractionation of the supernatant with $(NH_4)_2SO_4$ and successive column chromatographies on DEAE-cellulose, hydroxyapatite and Sepharose 6B. The chromatographic patterns on DEAE-cellulose and hydroxyapatite columns show two peaks of troponin T kinase activity. Gel filtration experiments indicate the existence of multiple, possibly aggregated, forms of the enzyme. The purified enzyme does not catalyze the phosphorylation of phosphorylase *b*, troponin I, troponin C, tropomyosin, protamine, or myosin light chain 2 nor does it catalyze the interconversion of glycogen synthase *I* into the *D* form. Troponin T kinase is not affected by the addition of cyclic nucleotides or AMP to the reaction mixture. Divalent cations (other than Mg^{2+}, required for the reaction) do not stimulate the enzyme, and several are inhibitory. Other characteristics of the reaction catalyzed by troponin T kinase, such as K_m values for ATP and substrate proteins, pH optima, effect of the concentration of Mg^{2+}, substitution of ATP for GTP have also been studied.

Introduction

Since the first report by Bailey and Villar-Palasi [1] on the covalent phosphorylation of troponin I by cyclic AMP-dependent protein kinase (ATP:protein phosphotransferase, EC 2.7.1.37), many investigations have been performed in the field of contractile protein phosphorylation. It has been shown that two sites of troponin I are phosphorylated by cyclic AMP-dependent pro-

tein kinase and phosphorylase *b* kinase [2—4], and that three sites of troponin T may be phosphorylated by phosphorylase *b* kinase [4,5]. However, the finding that extracts of muscle from mice with phosphorylase *b* kinase deficiency are able to phosphorylate troponin T [6] suggests the existence of a troponin T kinase distinct from phosphorylase *b* kinase.

The purpose of the present work was to study the chromatographic distribution of the protein kinases responsible for the phosphorylation of contractile proteins and their relationship to known protein kinases present in skeletal muscle. In the course of these studies, a new specific troponin T kinase was detected and purified free of phosphorylase *b* kinase and cyclic AMP-dependent protein kinase. The substrate specificity and other properties of this troponin T kinase were studied. This new protein kinase differs in its substrate specificity from the troponin T kinase recently isolated by Dobrovol'skii et al. [7].

Experimental procedures

Protein substrates. Tropomyosin and troponins T, I, and C were prepared from fresh rabbit muscle. After obtaining myofibrils by the method of Perry and Zydow [8], the troponin-tropomyosin complex was isolated by the method of Hartshorne and Mueller [9]. Troponins T, I, and C were purified by the methods described by Eisenberg and Kielley [10] and Greaser and Gergely [11]. Troponin T was also prepared by the method of Wilkinson [12]. Tropomyosin was isolated and purified by the procedure of Eisenberg and Kielley [10], followed by precipitation with Bailey's solution [13]. The purity of each preparation was determined by means of polyacrylamide disc gel electrophoresis [14] in the presence of sodium dodecyl sulfate (Fig. 1). In all cases, the troponin fractions were over 95% pure. The endogenous phosphate content of the isolated proteins was determined by a combination of the methods of Ames [15] and Itaya and Ui [16]. Tropomyosin, troponin T, troponin I, and troponin C contained 0.16, 0.50, 0.02 and 0.05 mol of total phosphate/mol of protein, respectively. Phosphorylase *b* was prepared from rabbit skeletal muscle according to the method of Fischer and Krebs [17]. Myosin light chain 2 was purified from rabbit skeletal muscle by the method of Holt and Lowey [18]. Calf thymus mixed histone (histone IIA), egg phosvitin, milk casein, α-casein, β-casein, γ-casein, salmon sperm protamine and bovine serum albumin were purchased from the Sigma Chemical Company.

Enzymes. Glycogen synthase *I* (purified by the method of Takeda et al. [19], specific activity 32 μmol glucose incorporated into glycogen/mg protein per min) and cyclic AMP-independent synthase *I* kinase (prepared essentially as described by Itarte et al. [20], specific activity 38 nmol ^{32}P incorporated into phosvitin/mg protein per 10 min) were gifts from Drs. P.J. Roach and A.A. de Paoli-Roach. Cyclic AMP-dependent protein kinase (purified according to Huang and Huang [21], specific activity 425 μmol ^{32}P incorporated into histone/mg protein per min) was donated by Dr. L.C. Huang.

Assay methods. Incubations were carried out at 30°C in a total volume of 100 μl. The composition of the assay mixtures was as follows: for cyclic AMP-dependent protein kinase: 0.1 mM [γ-^{32}P]ATP, 1 mg histone IIA/ml, 5 mM $MgCl_2$, 40 mM Tris-HCl (pH 7.5), 30 mM 2-mercaptoethanol, and 5 μM cyclic

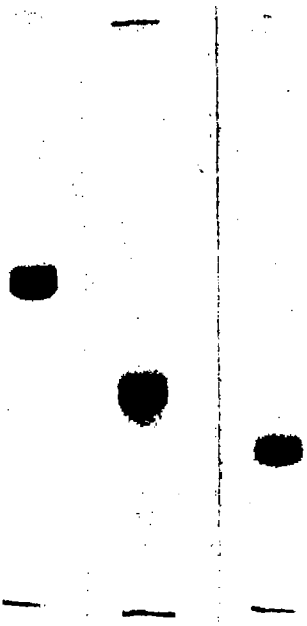

Fig. 1. Polyacrylamide (10% gels) gel electrophoresis in the presence of sodium dodecyl sulfate of samples of troponin T (left), troponin I (middle) and troponin C (right). 20 μg of each sample were applied to each gel. The protein bands were stained with Coomassie blue.

AMP (as indicated). For phosphorylase *b* kinase: 0.1 mM [γ-^{32}P]ATP, 1 mg phosphorylase *b*/ml, 10 mM $MgCl_2$, 90 mM Tris-HCl (pH 8.0), 30 mM 2-mercaptoethanol. The phosphorylation reaction mixtures for troponins, tropomyosin, phosvitin, casein and other protein substrates were as follows: 0.1 mM [γ-^{32}P]ATP, 5 mM $MgCl_2$, 30 mM Tris-HCl (pH 8.0), 90 mM KCl, 5 μM cyclic AMP (as indicated) and one of the following: 0.9 mg troponin T/ml, 1.1 mg troponin I/ml, 1.5 mg troponin C/ml, 1.1 mg tropomyosin/ml, 1 mg phosvitin, casein, or others/ml. The specific radioactivity of the [γ-^{32}P]ATP was 33—135 cpm/pmol, unless otherwise indicated. For the assay of the effects of ATP concentration of the activity, the specific radioactivity was 935 cpm/pmol. Each reaction was started by the addition of 10 μl enzyme solution diluted appropriately. All assays were linear and proportional to the amount of enzyme tested under these conditions. At the end of the incubation, 75-μl aliquots of the reaction mixtures were spotted onto 2 × 2 cm squares of Whatman ET 31 paper, which were then washed successively with 20% trichloroacetic acid, 10% trichloroacetic acid (twice), 5% trichloroacetic acid (twice) 50% ethanol/diethyl ether and diethyl ether alone. The washed papers were dried and placed in vials containing 10 ml of toluene/0.5% 2,5-diphenyloxazole scintillator. The radioactivity of the samples was measured with a liquid scintillation counter (Beckman, Model LS-3133). The degree of phosphorylation was calculated by subtracting radioactivity incorporated in each sample in the absence of substrate from the radioactivity incorporated in the presence of the substrate. For the assays of glycogen synthase interconversion, the following method was used. 5 μl of purified rabbit skeletal muscle synthetase *I* (equivalent to 10 μg

enzyme) were mixed with 25 μl of a solution containing 60 mM Tris-HCl, pH 7.8, 2 mM ATP, 12 mM $MgCl_2$, and 0.1 mM EGTA. To this mixture, one of the following was added: 10 μl of water, 10 μl of purified cyclic AMP-independent synthase *I* kinase (0.7—0.8 mg/ml), 10 μl of cyclic AMP-dependent protein kinase (1.1. mg/ml) containing 5 μM cyclic AMP, or 10 μl of purified troponin T kinase (1.2 mg/ml). Immediately after mixing at 0°C, and also after 30 and 60 min of incubation at 30°C, samples of 5 μl of each mixture were diluted with 500 μl of 50 mM Tris-HCl, pH 7.8, containing 20 mM KF, 5 mM EDTA, 2 mM EGTA and 1 mg rabbit liver glycogen/ml, at 0°C. Aliquots of these diluted samples were used for the assay of glycogen synthase activity by the method of Thomas et al. [22]. The decrease in the ratio of the activity in the absence of glucose 6-phosphate to that in the presence of this activator (originally 95—99%) was used as index of the interconverting activity of each enzyme preparation used.

Other methods. [γ-^{32}P]ATP was prepared by the method of Glynn and Chappell [23]. [γ-^{32}P]GTP (1.85 Ci/mmol) was purchsed from Amersham Corporation. Protein concentrations of the solutions of troponins T, I, and C were determined spectrophotometrically by the method of Eisenberg and Kielley [10], and those of other proteins by the method of Lowry et al. [24] with bovine serum albumin as standard.

Materials. DEAE-cellulose (DE-52) was purchased from Whatman. Hydroxyapatite (Bio-Gel HTP) was obtained from Bio-Rad Laboratories. Sepharose 6B and Ficoll were purchased from Pharmacia Fine Chemicals.

Results

Resolution of various protein kinases by column chromatography

All procedures were carried out at 4°C. 100 g fresh rabbit back muscle were ground and homogenized for 1 min in a Waring Blendor with 20 mM Tris-HCl, 2 mM EDTA, 15 mM 2-mercaptoethanol (pH 7.5, buffer 1). The homogenate was centrifuged at 20 000 ×*g* for 40 min and the supernatant fluid filtered through glass wool. The filtrate was diluted with an equal volume of glass distilled water and was applied to a DE-52 column (2.2 × 22 cm) previously equilibrated with buffer 1. After washing the column with the same buffer, elution was performed with a 600 ml linear concentration gradient of 0—300 mM NaCl in buffer 1. 7.5-ml fractions were collected and analysed for various protein kinase activities (Fig. 2). Histone phosphorylating activity was resolved into three peaks. The activities of the first and the third peaks were stimulated by cylic AMP (Fig. 2A); therefore, they correspond to the two cyclic AMP-dependent protein kinase (types I and II) isoenzymes. Phosphorylase *b* kinase appeared as a single, broad peak with a maximum coinciding with the second peak of cyclic AMP-dependent protein kinase (Fig. 2A).

The ability of the different fractions to catalyze the phosphorylation of tropomyosin and troponins T, I, and C was also tested (Fig. 2B). Tropomyosin and troponin C were not appreciably phosphorylated by any fraction. Troponin I phosphorylation was observed with two peaks, corresponding to the elution positions of the first cyclic AMP-dependent protein kinase and the peak containing both the second cyclic AMP-dependent protein kinase and phos-

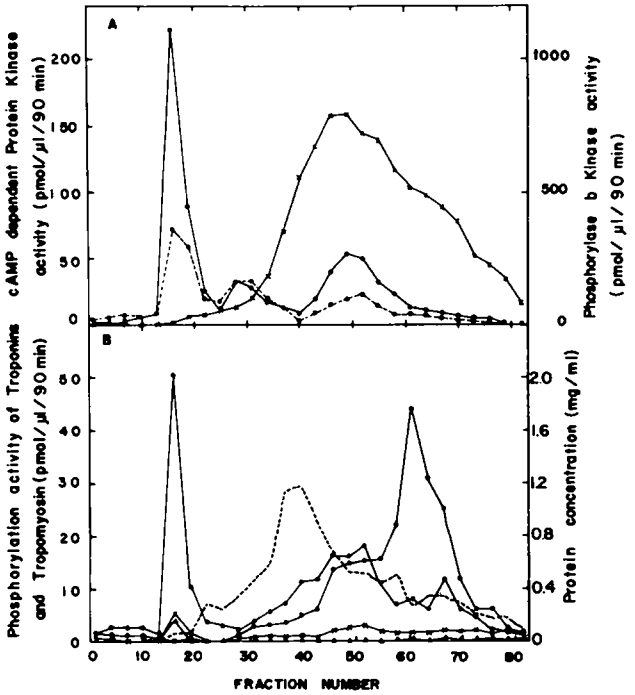

Fig. 2. DE-52 chromatography of rabbit skeletal muscle extract. (A) Activities of phosphorylase *b* kinase (X) and cyclic AMP-dependent protein kinase in the presence (•) and absence (○) of 5 µM cyclic AMP. For the assay, 10 µl of each fraction, diltued 1 : 10, were used. (B) Phosphorylation of troponin T (•), troponin I (in the presence of 5 µM cyclic AMP) (○), troponin C (△), and tropomyosin (X). Fractions diluted 1 : 5, 10 µl were used for the assay. The ordinate indicates pmol phosphate incorporated into each substrate/µl of undiluted eluate fraction after 90 min incubation. . . ., protein concentration.

phorylase *b* kinase. This is expected in view of the reports of troponin I phosphorylation by these two kinases [2—4]. Likewise, the phosphorylating activity towards troponin T was resolved into two regions, a small shoulder corresponding with the phosphorylase *b* kinase maximum, and a larger peak which eluted at a higher salt concentration (fraction 61). The small shoulder may be explained by the reported phosphorylation of troponin T by phosphorylase *b* kinase [4,5] but the main peak did not correspond with the maximum of any of the other kinases tested, suggesting, therefore, the existence of a specific kinase for troponin T.

Purification of troponin T kinase

Back muscle of three rabbits was ground and homogenized for 1 min in a Waring Blendor with ice-cold 4 mM EDTA (pH 7.0). All operations were carried out at 0—4°C. The homogenate was centrifuged at 4000 ×*g* for 40 min and the supernatant fluid was filtered through glass wool. The pH of the extract was adjusted to 6.1 by the dropwise addition of 1 N acetic acid; the suspension was stirred for 10 min and was centrifuged at 4000 ×*g* for 30 min. The pH of the resulting supernatant fluid was then adjusted to 7.0 with 1 M Tris base. Solid $(NH_4)_2SO_4$ was added slowly while stirring to bring the solution to 35% saturation; the mixture was maintained at pH 7.0 by addition of NH_4OH.

After stirring for 30 min, the suspensions was centrifuged at 4000 × *g* for 30 min. The supernatant fluid was brought to 70% saturation by the further addition of solid $(NH_4)_2SO_4$, while maintaining the pH at 7.0 with NH_4OH. After stirring for 30 min, the suspension was centrifuged at 4000 × *g* for 30 min. The precipitate was dissolved in 20 mM Tris-HCl (pH 7.5), 2 mM EDTA, 1 mM dithiothreitol, 0.15 M NaCl (buffer 2). After dialysis against two changes of buffer 2, the dialyzed sample was diluted with water in order to bring the conductivity of the solution to that of buffer 2. Cyclic AMP was added (final concentration 0.1 mM) to the diluted sample in order to dissociate the cyclic AMP-dependent protein kinase. This solution was then applied to a DE-52 column (4 × 25 cm) previously equilibrated with buffer 2/10 μM cyclic AMP (12.9-ml fractions). The column was washed with buffer 2/10 μM cyclic AMP and the enzyme eluted using a linear gradient of 0.15—0.3 M NaCl in 20 mM Tris-HCl (pH 7.5), 2 mM EDTA, 1 mM dithiothreitol (Fig. 3). The fractions 54–85, containing troponin T kinase activity, were combined and dialyzed four times against 20 mM potassium phosphate buffer (pH 7.5), 0.5 mM dithiothreitol (buffer 3). The dialyzed sample was applied to a hydroapatite column (1.53 × 17 cm) equilibrated with buffer 3 and the column was washed with the same buffer (3.9-ml fractions). Then, a linear 300 ml gradient of 20—250 mM potassium phosphate (pH 7.5), 0.5 mM dithiothreitol was applied to the column in order to elute the enzyme. Fractions 40—61 (Fig. 4) were combined

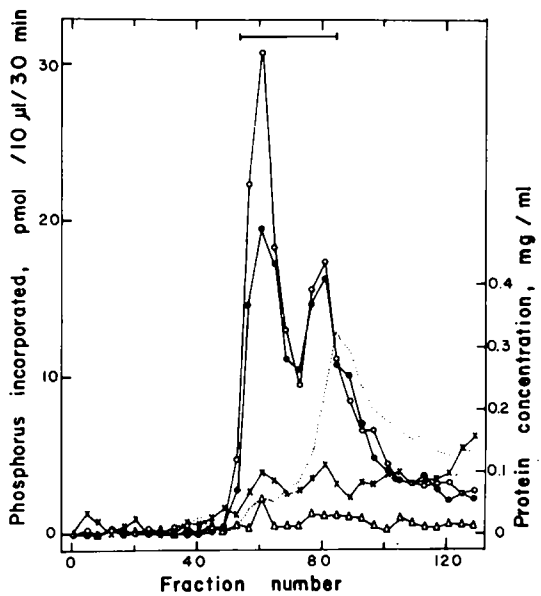

Fig. 3. DE-52 column chromatography of $(NH_4)_2SO_4$ fraction. The dialyzed $(NH_4)_2SO_4$ fraction (35—70%), with cyclic AMP added to a concentration of 0.1 mM, was applied to a 4.0 × 24 cm DE-52 column equilibrated with buffer 2/10 μM cyclic AMP. After the column had been washed with 1250 ml of buffer 2/10 μM cyclic AMP, the column was eluted with a 1700 ml linear gradient of 0.15—0.30 M NaCl in 20 mM Tris-HCl (pH 7.5) 2 mM EDTA, 1 mM dithiothreitol (12.9-ml fractions). Phosphorus incorporation into troponin T (●), phosvitin (○), phosphorylase *b* (×) and mixed histone (△) in the presence of 5 μM cyclic AMP/30 min was measured with the standard reaction mixture by employing 10 μl eluate. Protein concentration (- - - - - -) was determined by the method of Lowry et al. [24].

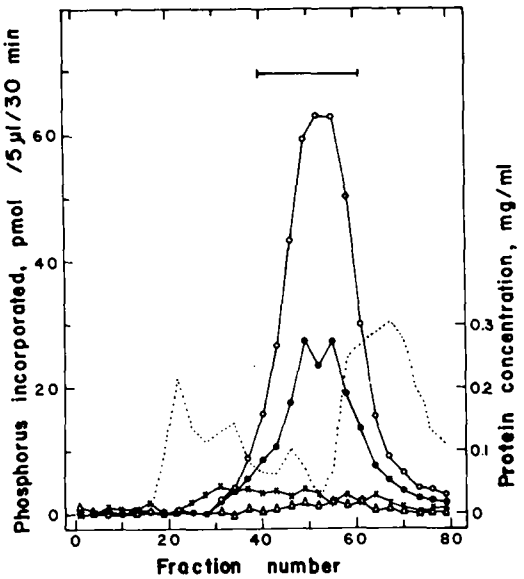

Fig. 4. Hydroxyapatite column chromatography of the DE-52 column fraction. The DE-52 column fractions 54—85 were combined, dialyzed and applied to a 1.5 × 17 cm hydroxyapatite column equilibrated with 20 mM potassium phosphate buffer (pH 7.5), 0.5 mM dithiothreitol. After washing the column with 130 ml of the above buffer, the column chromatography was performed by a 300 ml linear gradient of 20—250 mM potassium phosphate buffer (pH 7.5), 0.5 mM dithiothreitol (3.9-ml fractions). The assay mixture contained 5 µl eluate in the standard reaction mixture, and the incubation was carried out for 30 min. Phosphorus incorporated during 30 min incubation into troponin T (●), phosvitin (○), phosphorylase *b* (×), and mixed histone (△) in the presence of 5 µM cyclic AMP is shown respectively. Protein concentration also is given (- - - - - -).

and concentrated by dialysis against solid Ficoll and then against 20 mM potassium phosphate buffer (pH 7.5), 2 mM EDTA, 4 mM dithiothreitol, 50% glycerol. The concentrated enzyme (4.4 mg protein/ml) was stored at −20°C without freezing. This preparation was used for most of the kinetic studies. 50% of the activity was lost in 2 months under these conditions.

One portion of the concentrated enzyme (3.3 mg protein) was applied to a Sepharose 6B column (2.5 × 88 cm) equilibrated with 20 mM potassium buffer (pH 7.5), 1 mM dithiothreitol (3.9-ml fractions, flow rate 0.16 ml/min). Fractions 36—46, 47—52, 53—56, 57—62 and 63–68 were combined separately as Sepharose fractions I, II, III, IV, and V, respectively (Fig. 5). Troponin T kinase activity was accompanied in every fraction by phosvitin and casein phosphorylating activities. After polyacrylamide gel electrophoresis in the presence of SDS [14], the best purified enzyme fractions showed a main protein band, consistently present in every preparation and several faint bands. Therefore, these preparations were not homogeneous. Table I is a summary of the purification of troponin T kinase and the removal of other contaminating protein kinases.

Gel electrophoresis of Troponin T phosphorylated with purified troponin T kinase

Troponin T was incubated in the normal troponin phosphorylation reaction

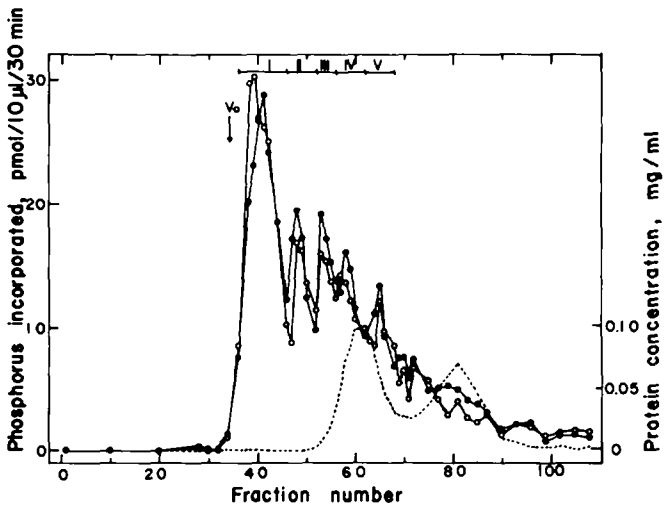

Fig. 5. Sepharose 6B gel filtration of the enzyme. The enzyme purified by hydroxyapatite column chromatography (24 nmol phosphorus incorporated into troponin T/min) was concentrated to 5 ml and 3.3 mg protein (6.7 nmol phosphorus incorporated into troponin T/min) was applied to a Sepharose 6B column (2.5 × 88 cm) equilibrated with 20 mM potassium phosphate buffer (pH 7.5), 1 mM dithiothreitol (3.9-ml fractions; flow rate 0.16 ml/min). Troponin T (•) and phosvitin (○) were incubated for 30 min with 10 μl eluate in the standard reaction mixture. Protein concentration is also given (- - - - -).

mixtured with [γ-^{32}P]ATP and purified troponin T kinase (hydroxyapatite column fraction). After incubation, the reaction mixture was analyzed by means of SDS-polyacrylamide gel electrophoresis (Fig. 6A). Essentially all the radioactivity incorporated was recovered in a protein band with a R_F = 0.45, corresponding to troponin T. A very minor radioactivity band (migrating at a different R_F) was also present in the control incubated without troponin T (Fig. 6B), and, therefore, represents phosphorylation of a protein present in the troponin T kinase preparation and can be discounted.

Time course of troponin T and phosvitin phosphorylation

In order to better define assay conditions, the time courses of troponin T (Fig. 7A) and phosvitin (Fig. 7B) phosphorylation were determined using various concentrations of troponin T kinase. When less than 44 μg/ml of enzyme were used the amount of phosphorus incorporated into the substrates was proportional to the incubation time for up to 10 min. However, incubation for longer than 10 min resulted in a decreased rate of phosphorylation of both substrates. This was especially marked in the case of troponin T phosphorylation. The decrease in reaction rate appeared to be due to a change in the enzyme activity, rather than to the exhaustion of any other component of the reaction mixture, because (a) the time couse of phosphorylation by even the lowest concentration of enzyme used (0.9 μg/0.1 ml) showed this behavior, and (b) because further addition of enzyme later during the incubation increased the ^{32}P incorporation into the substrate protein. Incorporation of radioactivity into proteins present in the enzyme preparation itself was negligible. Up to 0.15 mol ^{32}P phosphate was bound/mol of troponin T (over the endogenous 0.5 mol/mol). It is not yet clear whether this is the true maximum value.

TABLE I

SUMMARY OF THE PURIFICATION OF TROPONIN T KINASE

Activities were determined in each case under conditions where phosphorylation was proportional to enzyme concentration and time of incubation (10 min). Troponin I and mixed histone (+cAMP) phosphorylating activities were measured in the presence of 5 μM cyclic AMP. All Sepharose 6B fractions were concentrated before the determinations of protein concentrations and enzyme activites.

Fractions	Total protein (mg)	Phosphorus incorporated (nmol/min per fraction)(yield, %) in:					
		Troponin T	Troponin I	Phosvitin	Phosphorylase	Mixed (+cAMP)	Histone (−cAMP)
1. Crude Extract	34 500	264 (100)	227 (100)	363 (100)	86 300 (100)	5 100 (100)	1 890 (100)
2. Supernatant at pH 6.1	33 900	97 (37)	200 (88)	217 (60)	31 000 (36)	6 400 (125)	2 210 (117)
3. $(NH_4)_2SO_4$ fraction (35–70%)	14 900	52 (20)	6 (3)	80 (22)	10 (0.012)	2 420 (47)	1 000 (53)
4. DE-52 column fraction passed through	13 000	<0.3	5 (2)	48 (13)	8 (0.009)	2 600 (51)	2 830 (150)
Fraction washed out	307	<0.5	<0.5	6 (2)	6 (0.007)	122 (2)	116 (6)
Fractions 54–85	60	44 (17)	<0.2	23 (6)	1 (0.001)	5 (0.1)	5 (0.3)
5. Hydroxyapatite column fractions 40–61	12	24 (9)	<0.2	17 (5)	0.4 (0.0005)	0.7 (0.01)	0.7 (0.04)
6. Sepharose 6B column:							
Fraction I	0.5	8.3 (3.1)	<0.1	5.3 (1.5)	<0.1	0.3 (0.006)	0.3 (0.016)
Fraction II	0.4	2.8 (1.1)	<0.1	2.0 (0.6)	<0.1	<0.1	<0.1
Fraction III	0.6	1.8 (0.7)	<0.1	1.3 (0.4)	<0.1	<0.1	<0.1
Fraction IV	3.7	2.2 (0.8)	<0.1	1.4 (0.4)	<0.1	<0.1	<0.1
Fraction V	1.4	0.9 (0.3)	<0.1	0.8 (0.2)	<0.1	<0.1	<0.1

Phosphorylation of troponin T was proportional to enzyme concentration of up to 2.2 μg/0.1 ml. With phosvitin as substrate, proportionality was observed even at 4.4 μg of enzyme/0.1 ml. All experiments described were performed under conditions where the reaction rate was proportional to time and enzyme concentration.

pH optima

The optimum pH for troponin T phosphorylation was 8.0; with phosvitin as substrate it was 7.0, both with rather broad activity-pH profiles.

Effects of protein substrate concentration on the activity

The relationship between troponin T and phosvitin concentrations and phosphorylating activity of the purified enzyme is shown in Fig. 8. The apparent

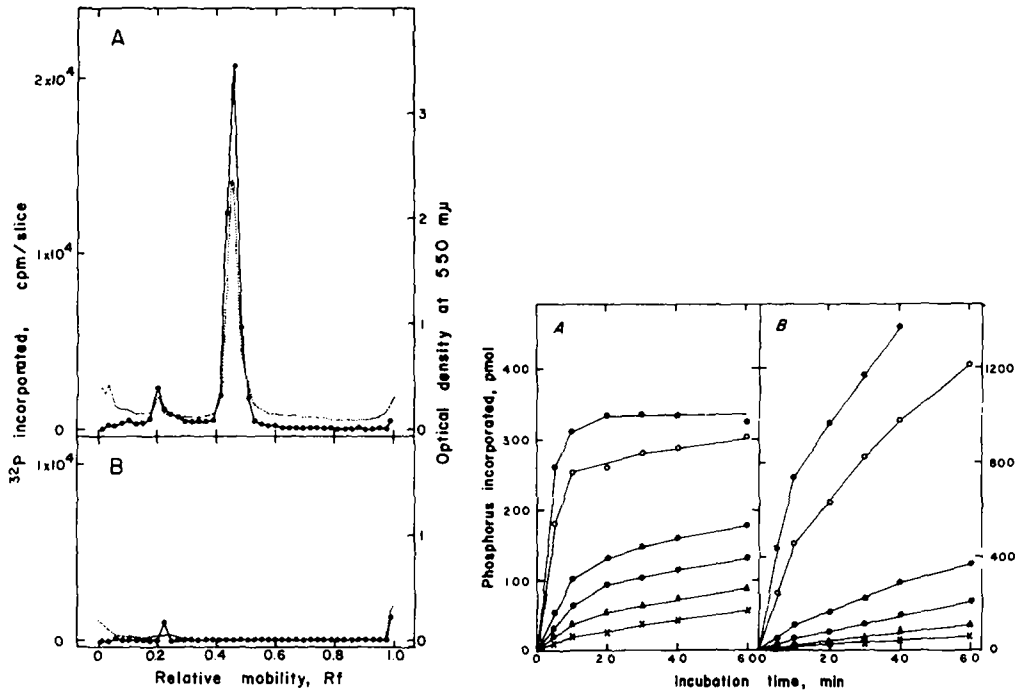

Fig. 6. SDS-polyacrylamide disc gel electrophoresis of phosphorylated troponin T. The phosphorylation mixture was 60 mM Tris-HCl (pH 8.0), 30 mM 2-mercaptoethanol, 0.1 mM [γ-^{32}P]ATP (3130 cpm/pmol), 20 mM $MgCl_2$; 4.4 μg purified troponin T kinase and either 64 μg troponin T (A) or no substrate (B). (Total volume, 100 μl; for 60 min at 30° C.) The reactions were stopped by the addition of 400 μl of sample buffer (1% SDS, 1% 2-mercaptoethanol, 10 mM sodium phosphate, 6 M urea, pH 7.2). After 10 min in a boiling water bath, 100 μl of each sample (containing 5 μl 0.5% bromphenol blue) were applied per gel. ●———●, radioactivity incorporated; absorbance (· · · · · ·), absorbance of stained bands.

Fig. 7. Time course of troponin T and phosvitin phosphorylation by various amounts of enzyme. The phosphorylations of troponin T (A) and phosvitin (B) were measured by the enzyme amounts of 0.9 (×), 1.3 (△), 2.2 (●), 4.4 (⊖), 22 (○), and 44 (◑) μg in the regular reaction mixture (0.1 ml).

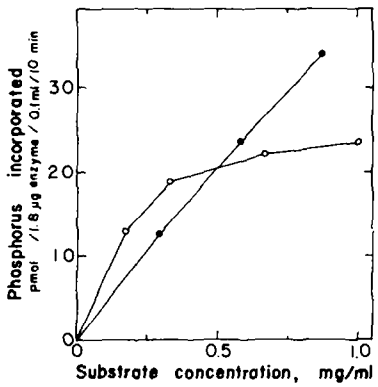

Fig. 8. The effects of substrate protein concentration on phosphorus incorporation into troponin T and phosvitin. Troponin T (●) of 0.29, 0.58, and 0.87 mg/ml, and phosvitin (○) of 0.17, 0.33, 0.67 and 1.0 mg/ml were incubated for 10 min with 1.8 μg enzyme in the regular reaction mixture (0.1 ml).

K_m for phosvitin was 0.2 mg/ml; therefore, the phosvitin concentration used in the standard assay mixture (1 mg/ml) was nearly saturating. On the other hand, troponin T phosphorylation was proportional to the concentration of troponin T added up to a concentration of 0.87 mg/ml. Since troponin T is relatively insoluble in the test mixture, it was not feasible to determine its K_m under the conditions used.

Substrate specificity

Protein substrate specificity studies of troponin T kinase are presented in Table II. Of all the skeletal muscle proteins tested, only troponin T was phosphorylated. Acidic proteins, such as phosvitin and caseins, were also phosphorylated.

The effects of troponin T kinase on the activity of glycogen synthase were tested as follow: the phosvitin phosphorylating activity of troponin T kinase and cyclic AMP-independent synthase *I* kinase [19] were measured. Amounts of each enzyme having equivalent phosvitin phosphorylating activity (30 pmol/min at 30°C, in 10 μl) were tested as described in the Experimental procedures. A blank without added enzyme (to correct for a small amount of endogenous kinase activity present in the synthase *I* preparation) was incubated in parallel. Cyclic AMP-dependent protein kinase was also tested simultaneously as a control. Under these conditions, cyclic AMP-independent synthase *I* kinase was able to decrease the per cent synthase *I* from 99% to 42% in 60 min. The decrease observed with cyclic AMP-dependent protein kinase was from 97% to 23%. However, troponin T kinase did not catalyze any conversion above that of the water blank (from 95% to 87% in 60 min). Troponin T kinase,

TABLE II

SUBSTRATE SPECIFICITY OF TROPONIN T KINASE

The indicated amounts of substrate listed in the table were incubated for 10 min with 0.9 μg enzyme in the regular reaction mixture for troponin T. Relative phosphorylation activity was expressed % of troponin T phosphorylation.

Substrate (μg/assay)	Relative activity (%)
Troponin T (87)	100
Phosvitin (100)	83
α-Casein (100)	52
Casein (100)	39
γ-Casein (100)	18
β-Casein (100)	10
Mixed histone *	8
Mixed histone (100) **	4
Tropomyosin (108)	4
Troponin I (102 **	0
Troponin C (98)	0
Myosin light chain 2 (100)	0
Protamine (100)	0
Phosphorylase *b* (100)	0
Serum albumin (100)	0

* Mixed histone phosphorylation in the absence of cyclic AMP.

** Mixed histone or traponin I phosphorylation in the presence of 5 μM cyclic AMP.

therefore, was unable to catalyze the interconversion of glycogen synthase *I* into the *D* form.

Effects of the concentration of ATP and GTP on the kinase activity

The phosphorylation of troponin T and phosvitin was tested using several concentrations of ATP. When troponin T was the substrate, the reaction followed Michaelis-Menten kinetics, with some inhibition at high ATP concentrations. The apparent K_m for ATP was 35 μM. With phosvitin as substrate, the kinetic results did not fit a hyperbola and no clear saturation was observed. From double reciprocal plots, two apparent K_m values, 25 μM and 400 μM, could be extrapolated from linear segments of the plot. The molecular basis for this non-hyperbolic behavior, however, remains to be established.

Since several phosphoproteins from various sources are phosphorylated by kinases able to utilize GTP [25—29], the ability of GTP to substitute for ATP as phosphoryl donor was investigated. When GTP (at concentrations from 0.05 to 15 mM) was used instead of ATP in the standard reaction mixture, no phosphate incorporation into either troponin T or phosvitin was observed.

Heat stability of the enzyme

Phosphorylating activity towards troponin T, phosvitin and casein was completely lost by preincubating the enzyme for 30 min at 60°C. Heating the enzyme for 30 min at 45°C resulted in a 10% decrease of these activities. 40 min preincubation of the enzyme at 50°C resulted in 67, 70, and 81% loss of the original troponin T, casein and phosvitin phosphorylating activities, respectively. Troponin T and casein phosphorylating activities were, therefore, more stable to thermal denaturation than was the phosvitin phosphorylating activity.

Effects of divalent cations and EDTA on the phosphorylating activity

The effects of the concentration of Mg^{2+} on troponin T and phosvitin phosphorylation were studied. The maximal activities towards troponin T and phosvitin were observed at 5 and 1 mM of added magnesium chloride, respectively. In the case of phosvitin, phosphorylation was observed without addition of Mg^{2+} to the reaction mixture. The same result was obtained using phosvitin dialyzed successively against 5 mM EDTA, pH 8.0 (once, 4 h) and 5 mM Tris-HCl, pH 8.0 (twice, 4 h each time) (Fig. 9). The phosphorylation of phosvitin seems to require Mg^{2+}, however, because addition of 10 mM EDTA to the standard reaction mixture containing 5 mM $MgCl_2$ inhibited completely not only troponin T phosphorylation but also phosvitin phosphorylation. Clark [30] has reported that egg phosvitin is contaminated by Mg^{2+} (10—139 g magnesium/10^4 g phosvitin) and that it is very difficult to remove this contamination by dialysis. Therefore, the phosphorylation of phosvitin in the absence of added Mg^{2+} may be due to the presence of this metal in the phosvitin preparation itself. Table III shows the effects of several divalent cations (0.5 mM) added to the standard assay on troponin T and phosvitin phosphorylations. None of them had any stimulatory effect on either phorphorylation reaction, and, indeed, $MnCl_2$, $ZnCl_2$, or $HgCl_2$ inhibited both reactions. Moreover, none of the divalent cations listed in Table III (at 5 mM concentrations) could substitute for 5 mM Mg^{2+} in the standard reaction mixture.

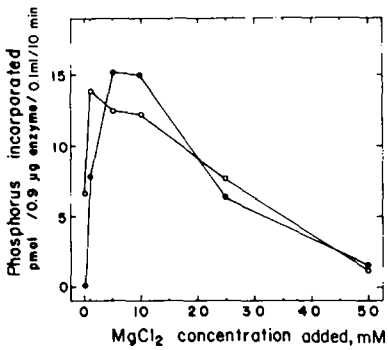

Fig. 9. The effects of $MgCl_2$ concentrations on troponin T and phosvitin phosphorylations. Phosvitin was used after dialyzing it against 5 mM EDTA (pH 8.0) (once, 4 h) and 5 mM Tris-HCl (pH 8.0) (twice, 4 h each). Troponin T (●) and phosvitin (○) were incubated for 10 min with 0.9 µg enzyme in the regular 0.1 ml reaction mixture containing 0—50 mM of external $MgCl_2$.

Effects of nucleotides and other compounds on troponin T and phosvitin phosphorylations

Cyclic AMP, cyclic GMP, cyclic IMP, cyclic UMP, and cyclic CMP, at concentrations from 1 to 100 µM, and AMP from 10 µM to 1 mM had no effect on either the phosphorylation of troponin T or phosvitin. Addition of ADP to the standard reaction mixture containing 0.1 mM ATP resulted in clear inhibition of the phosphorylation of troponin T and phosvitin. At 0.1 mM ADP, the inhibition was 30—40%, and at 1.0 mM ADP the inhibition reached 80—100%, depending on the protein substrate used. Dithiothreitol (1—50 mM), 2-mercaptoethanol (1—100 mM), and *N*-ethylmaleimide (1—5 mM) had no effect on either the phosphorylation of troponin T or phosvitin. Potassium fluoride produced a 20% inhibition at 10 mM, and at 50 mM the inhibition observed reached 85—90%.

TABLE III

THE EFFECTS OF DIVALENT CATIONS ON TROPONIN T AND PHOSVITIN PHOSPHORYLATION ACTIVITY

Various kinds of cation chlorides (0.5 mM) were added to the regular reaction mixture containing 5 µM $MgCl_2$ and troponin T and phosvitin phosphorylation reactions were performed for 10 min with 0.5 µg enzyme.

Cation (0.5 mM)	Relative activity (%)	
	Troponin T	Phosvitin
Control	100	100
$SrCl_2$	106	89
$BaCl_2$	104	63
$CuCl_2$	83	63
$CoCl_2$	66	87
$CaCl_2$	64	93
$MnCl_2$	8	30
$ZnCl_2$	5	0
$HgCl_2$	0	8

Discussion

The distribution of cyclic AMP-dependent protein kinase, phosphorylase *b* kinase, and troponin and tropomyosin phosphorylating activities after chromatography (Fig. 2) supports the reports that troponins I and T are phosphorylated by cyclic AMP-dependent protein kinase and/or phosphorylase *b* kinase, while troponin C and tropomyosin are not phosphorylated [31]. However, the major troponin T phosphorylating activity peak was eluted later than the maximal activity of phosphorylase *b* kinase. This finding pointed to the possible phosphorylation of troponin T by an enzyme distinct from phosphorylase *b* kinase. We have succeeded in the purification of a troponin T phosphorylating enzyme, tentatively designated as troponin T kinase. The purified preparations of this new kinase are free of phosphorylase *b* kinase and cyclic AMP-dependent protein kinase (Table I).

The crude muscle extract catalyzed the phosphorylation of troponin T, troponin I, phosvitin, phosphorylase *b*, and mixed histone. The activities towards these substrates were measured at every step of the purification. About $\frac{2}{3}$ of the original phosphorylase *b* kinase activity of the crude extract was removed in the pH 6.1 precipitate. Almost all of this remaining activity was recovered in the precipitate obtained at 35% saturation of $(NH_4)_2SO_4$ (not shown).

About 20% of the troponin T phosphorylating activity of the crude extract was retained on the DE-52 column. Under these conditions, the dissociated catalytic subunit of the cyclic AMP-dependent protein kinase was eluted in the pass-through and washout fractions. Troponin T kinase activity was eluted as two peaks of activity (Fig. 3). When the pooled fractions were applied to the hydroxapatite column and eluted, troponin T kinase activity again appeared in two peaks (Fig. 4). This elution pattern suggests the possible existence of two molecular species of the enzyme. On the other hand, troponin T kinase was eluted from the Sepharose 6B column in at least 5 peaks, which most likely represent molecular aggregates.

Phosvitin phosphorylating activity coincided with troponin T phosphorylating activity in the fraction obtained after DE-52 and hydroxyapatite chromatographies (Figs. 3 and 4) and Sepharose 6B gel filtration (Fig. 5). In every case troponin T kinase was also accompanied by casein-phosphorylating activity (not shown in the figures). Thus, these appear closely related activities.

The specific troponin T phosphorylating activities of the crude extract and the main peak on Sepharose 6B gel filtration were 7.7 and 16 600 pmol/mg protein per min, respectively. Thus, troponin T kinase was purified by at least 2156 fold (not correcting for the troponin T phosphorylating activity of phosphorylase *b* kinase) by the methods described here.

An evaluation of the relative troponin T phosphorylating activities of troponin T kinase and phosphorylase *b* kinase can be tentatively obtained from the data presented. After essentially complete removal of phosphorylase *b* kinase from the preparations (Table I, 35—70% ammonium sulfate saturation step), there remains 20% of the original troponin T phosphorylating activity. Furthermore, examination of the elution pattern of the crude extract on DE-52 (Fig. 2) shows that the peak of phosphorylase *b* kinase activity coincided with

a small shoulder of troponin T phosphorylating activity. The main peak of troponin T phosphorylating activity eluted at higher salt concentrations (Fig. 2), coinciding with the elution pattern of troponin T kinase (Fig. 3). Therefore, it can be concluded that certainly no less than 20%, and probably over 59%, of the total troponin T phosphorylating activity in skeletal muscle can be accounted for by the troponin T kinase activity.

Skeletal muscle appears to contain several protein kinases able to catalyze the phosphorylation of egg phosvitin [20]. One of these protein kinases, the major phosvitin kinase in muscle [32], catalyzes the phosphorylation of glycogen synthase *I* and its conversion into the *D* form [20,32,33], and, therefore, may have a physiological function. From the data presented in this paper it appears clear, however, that troponin T kinase and this glycogen synthase *I* kinase are different enzymes. Purified troponin T kinase was unable to catalyze the conversion of glycogen synthase *I* into the *D* form. Also, troponin T kinase was eluted from the DEAE-cellulose columns at a concentration of 0.22—0.25 M NaCl while higher salt concentrations (over 0.5 M NaCl) are required to elute synthetase *I* kinase [33]. Troponin T phosphorylating activity was as stable to thermal inactivation as casein phosphorylating activity, while glycogen synthase *I* phosphorylating activity is either more heat labile [33], or does not phosphorylate casein [32].

Finally, there is the question of the identity of the troponin T kinase reported here and that of Dobrovol'skii et al. [7]. There is only preliminary information about the characteristics of their protein kinase. Their data show, however, that histone is as good or better substrate than troponin T for their protein kinase, while the troponin T kinase purified by our method had little or no phosphorylating activity towards histone. The possible role of this new troponin T kinase must await further study.

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