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Isolation and some properties of troponin T kinase from rabbit skeletal muscle

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A method for isolation of troponin T kinase (ATP–protein phosphotransferase, EC 2.7.1.37) from rabbit skeletal muscles is proposed. The method gives a 7000–10000-fold purification and results in an enzyme with specific activity of 400–800 nmol·min^{-1}·mg^{-1} of protein. The molecular weight of troponin T kinase as determined by gel filtration exceeds 500000. Electrophoresis in polyacrylamide gel in the presence of sodium dodecyl sulphate revealed that isolated preparations of the enzyme consisted of at least three distinct proteins with apparent mol.wts. of 50000, 46000 and 31000. The enzyme phosphorylates isolated troponin T at a rate which exceeds the phosphorylation rates of casein, phosvitin, histones, phosphorylase *b* and protamine 5–30-fold. Within the whole troponin complex, only troponin T is phosphorylated by the enzyme. The enzyme phosphorylates only the *N*-terminal serine residue of troponin T, i.e. the site that is normally phosphorylated in the whole troponin complex isolated from rabbit skeletal muscles.

Since the first publication by Bailey & Villar-Palasi (1971), who demonstrated that troponin I was readily phosphorylated by cyclic AMP-dependent protein kinase, many investigations have been carried out on troponin phosphorylation (Stull *et al.*, 1972; Pratje & Heilmeyer, 1972; Perry & Cole, 1973, 1974; Perry *et al.*, 1975). Great progress has been made in the study of phosphorylation of the whole troponin complex from the heart.

At present there are extensive data on an enzyme that phosphorylates heart troponin, on a component of troponin which is phosphorylated by cyclic AMP-dependent protein kinase, on a site of troponin I phosphorylation, etc. (Cole & Perry, 1975; Moir & Perry, 1977; England, 1975, 1976; Ray & England, 1976).

Much progress has been made in elucidation of the physiological role of heart troponin phosphorylation (England, 1976; Solaro *et al.*, 1976; Wyborny & Reddy, 1978; Holroyde *et al.*, 1979). Up to now, the phosphorylation of skeletal-muscle troponin has not been studied in detail. It is known that among skeletal-muscle troponin components the extent of troponin T phosphorylation is the highest. Usually it contains about 1 mol of phosphate/mol (Perry & Cole, 1974; Cole & Perry, 1975). The cyclic AMP-dependent protein kinase phosphorylates the skeletal muscle troponin at a very low rate and incorporates only 0.05–0.1 mol of phosphate/mol of protein (Pratje & Heilmeyer, 1972; Korovkin & Antipenko, 1979). This fact proves that the cyclic AMP-dependent protein kinase is unlikely to participate in the phosphorylation of troponin T *in vivo*.

Three sites in the troponin T structure (serine-1, serine-149 or serine-150 and serine-156 or serine-157) can be phosphorylated by purified preparations of phosphorylase *b* kinase (Moir *et al.*, 1977; Pearlstone *et al.*, 1976). However, only the *N*-terminal serine residue is phosphorylated in troponin T isolated from skeletal muscle. The rate of troponin phosphorylation is many times less than that of phosphorylase *b* phosphorylation, and the phosphorylation-rate ratio of troponin T to phosphorylase *b* varies for preparations of phosphorylase *b* kinase (Perry *et al.*, 1975; Dickneite *et al.*, 1978).

On the basis of these data it was assumed that the troponin T kinase is present in skeletal muscles (Dobrovolskii *et al.*, 1976; Gusev *et al.*, 1978). Kumon & Villar-Palasi (1979) have also reported the isolation of troponin T kinase from rabbit skeletal muscles.

The present paper describes the purification

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procedure and characterizes some properties of troponin T kinase. Some aspects of the work have been briefly reported (Gusev & Dobrovolskii, 1978).

Materials and methods

Troponin was isolated from rabbit skeletal muscles by the method of Staprans *et al.* (1972), with small modifications described previously (Dobrovolskii & Gusev, 1978). Troponin components were purified as described by Perry & Cole (1974) and Wilkinson (1974). The dephosphorylated troponin complex was obtained by mixing equimolar amounts of troponin C, troponin I and dephosphorylated troponin T, dissolved in 0.1 M-potassium phosphate buffer, pH 7.0, containing 8 M-urea, 2 mM-EDTA and 2 mM-2-mercaptoethanol, with subsequent dialysis against water and freeze-drying.

Histone H1 and phosvitin were isolated from calf thymus and egg yolk as described by Johns & Butler (1962) and Joubert & Cook (1958) respectively. Histones H2a, H2b, H3 and H4 were kindly supplied by Professor E. S. Severin (Moscow, U.S.S.R.). Protamine and casein were purchased from Chemapol (Czechoslovakia) and Soyuzkhimreaktiv (U.S.S.R.) respectively.

Alkaline phosphatase from *Escherichia coli* was isolated by the method of Garen & Levinthal (1960) and immobilized on CNBr-activated Sepharose 4B in 0.1 M-sodium tetraborate/orthoboric acid buffer, pH 8.0, for 4–5 h. Dephosphorylation of isolated troponin T by immobilized phosphatase was performed in 1 M-Tris/HCl (pH 8.0)/10 mM-$MgCl_2$ for 4–6 h at room temperature (18–20°C). Dephosphorylated troponin T was further purified and concentrated on CM-cellulose (Wilkinson, 1974) and immobilized on CNBr-activated Sepharose 4B in 0.1 M-$NaHCO_3$/1 M-NaCl for 4–5 h. Approx. 6 mg of protein was coupled to gel produced from 1 g of Sepharose.

The activity of protein kinase was determined by incorporation of ^{32}P from [γ-^{32}P]ATP into the protein substrate. The assay mixture contained: 50 μl of buffer [25 mM-KH_2PO_4/16.5 mM-Tris (pH 7.2)/10 mM-magnesium acetate/2 mM-dithiothreitol], 5 μl of [γ-^{32}P]ATP (2.5 nmol of ATP, 1×10^5–2×10^5 c.p.m.), 5–10 μl of protein kinase (0.01–1 mg/ml), 25 μl of protein substrate solution (approx. 1 mg/ml) and water up to 100 μl. Since in low-ionic-strength solutions troponin T is only slightly soluble at neutral and alkaline pH, the troponin T solution was prepared in double-distilled water, and the protein kinase reaction was started by addition of troponin T to the reaction mixture. The reaction was measured at 30°C for various time intervals. The amount of the enzyme in the assay mixture was chosen so that the reaction was linear for at least 5–10 min. To determine the pH optimum for protein kinase, the assay buffer was titrated with solid Tris until the desired pH was reached. The incorporation of ^{32}P into the protein was determined by the method of Reimann *et al.* (1971) or by the modification (Gusev *et al.*, 1978) of the Witt & Roskoski (1975) procedure.

Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis was performed in the homogeneous (Weber & Osborn, 1969) and heterogeneous (Schelud'ko, 1974) systems. All samples and molecular-weight markers were reduced before application to the gel (Weber & Osborn, 1969). The molecular weights were obtained from a calibration curve, which was constructed by assuming the following apparent mol.wts.: 18000 for troponin C, 22000 for troponin I, 38000 for troponin T, 42000 for ovalbumin, 68000 for bovine serum albumin and 98000 for phosphorylase *b*.

Polyacrylamide-gel electrophoresis in the absence of denaturing agents was performed as described by Schaub & Perry (1969). Isoelectrofocusing in polyacrylamide gel was carried out as described by Pires & Perry (1977); the activity of protein kinase was determined after extraction overnight of 2 mm-wide gel slices by 200 μl of the assay buffer. The gels were stained (Weber & Osborn, 1969) with Coomassie Brilliant Blue R-250, and after destaining were scanned in a Pye–Unicam SP. 1700 spectrophotometer.

To determine the site of troponin T phosphorylation, ^{32}P-labelled troponin T was digested with trypsin at the weight ratio trypsin/troponin T 1:50 in 0.1 m-Tris/HCl, pH 7.4, for 4 h at 30°C. The tryptic digest was then subjected to gel filtration on a column (1.2 cm × 100 cm) of Bio-Gel P-4, equilibrated with 0.1 M-HCl. The radioactive peptide obtained after gel filtration was hydrolysed by Pronase (Calbiochem) at the weight ratio peptide/enzyme 25:1; the pH of the reaction medium was adjusted continuously to pH 8.0 with NH_3. The mixture of peptides obtained was applied to a Dowex 50 (X 2; H^+ form) column (1.5 cm × 10 cm). The radioactive material, which was not adsorbed by the cation exchanger and was eluted by water, was hydrolysed in vacuum in 6 M-HCl at 105°C for 24 h and subjected to analysis in a Hitachi 034 KLA-3 amino acid analyser.

Protein concentration was determined either with Amido Black (Schaffner & Weismann, 1973) or as described by Lowry *et al.* (1951), with bovine serum albumin (Reanal, Hungary) as standard. The concentrations of the troponin components were determined from the molar absorption coefficients for troponin T (5.0), troponin I (3.8) and troponin C (1.4) (Staprans *et al.*, 1972).

Materials

The chromatographic media, i.e. DEAE-cellulose

(DE-52), CM-cellulose (CM-32) and ion-exchange paper (P-81), were obtained from Whatman; cellulose phosphate (P-11) and Tris were from Sigma. 5'-AMP–Sepharose, CNBr-activated Sepharose and Sephadex G-200 were purchased from Pharmacia Fine Chemicals. Bio-Gel P-4 was obtained from Bio-Rad Laboratories. Hydroxyapatite was synthesized as described by Bernardi (1971). Ampholine pH 3–10 was from LKB; ATP was from Reanal (Hungary). [γ-^{32}P]ATP was from The Radiochemical Centre, Amersham, Bucks., U.K. All the other reagents were purchased from Soyuzkhimreactiv.

Results

Isolation of crude preparations of protein kinase

All operations were performed at 4°C. Minced skeletal muscles of the rabbit were homogenized for 2min in 2.5vol. of 4mM-EDTA, pH7.5, and centrifuged at 3000*g* for 20min; 1.5M-potassium phosphate buffer, pH7.0, was added to the supernatant to a final concentration of 30mM. The solution obtained was adjusted to 30% saturation with solid $(NH_4)_2SO_4$. The precipitate formed was removed by centrifugation at 9000*g* for 20min. $(NH_4)_2SO_4$ was then added to the supernatant to 53% saturation. After standing for 30min, the precipitate was collected by centrifugation at 9000*g* for 20min, suspended in buffer A (50mM-potassium phosphate/2mM-EDTA/2mM-2-mercaptoethanol, pH7.0), and dialysed against 30vol. of buffer A overnight. Insoluble and denatured proteins were removed by centrifugation at 105000*g* for 1h, and the supernatant (the crude preparations of protein kinase) was either used immediately for the further purification or was frozen at −20°C in the presence of 10% (w/v) sucrose if necessary for storage.

Purification of troponin T kinase

The crude preparation of protein kinase was applied to a DEAE-cellulose column equilibrated against buffer A (Fig. 1). The column was washed with buffer A until the A_{280} of the eluate was below 0.2 and then eluted with 0.15M-potassium phosphate buffer, pH7.0, containing 2mM-EDTA and 2mM-2-mercaptoethanol. On application of a linear gradient of 0–0.25M-NaCl in the last buffer, troponin T kinase was eluted in a peak corresponding to 0.12M-NaCl.

The fractions representing the eluted peak of troponin T kinase were pooled and dialysed against 3vol. of 2mM-EDTA/2mM-2-mercaptoethanol, pH7.0 (buffer B), for 3h with vigorous stirring, and applied to a hydroxyapatite column (Fig. 2) equilibrated against buffer A. The column was washed with buffer A until the A_{280} of the eluate was zero and then the column was eluted with a gradient of 0.05–0.25M-potassium phosphate in buffer A. The proteins that were not adsorbed or only slightly adsorbed under these conditions possessed no troponin T kinase activity. The enzyme was eluted when the concentration of the phosphate buffer was

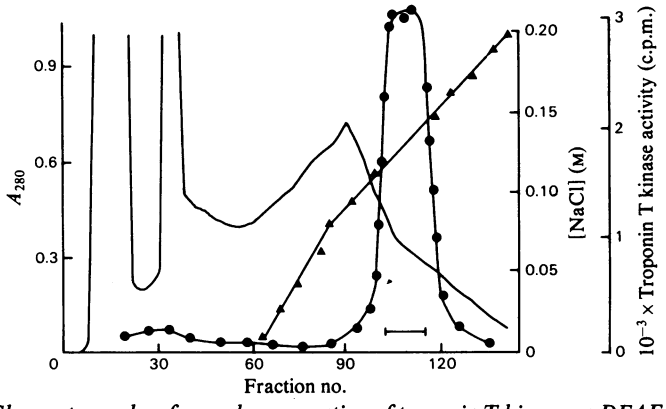

Fig. 1. *Chromatography of a crude preparation of troponin T kinase on DEAE-cellulose*

Crude preparation of troponin T kinase (300ml; 17.8mg/ml) was applied to a column (2.5cm × 30cm) of DEAE-cellulose DE-52 equilibrated against buffer A. After unadsorbed materials had been washed out with buffer A, the column was eluted successively with 0.15M-potassium phosphate, pH7.0 (1 bed vol.), and with a 1400ml linear gradient of 0–0.25M-NaCl in 0.15M-potassium phosphate buffer, pH7.0. All solutions used contained 2mM-EDTA and 2mM-2-mercaptoethanol. Fractions were collected every 9min at a flow rate of 100ml/h. Portions (5μl) of fractions were investigated for troponin T kinase activity by using the standard assay system (see the Materials and methods section). ——, A_{280}; ●, activity of troponin T kinase (amount of ^{32}P incorporated in troponin T in 5min per 5μl of a fraction); ▲, NaCl gradient monitored by conductivity measurements on the collected fractions; |——|, pooled fractions.

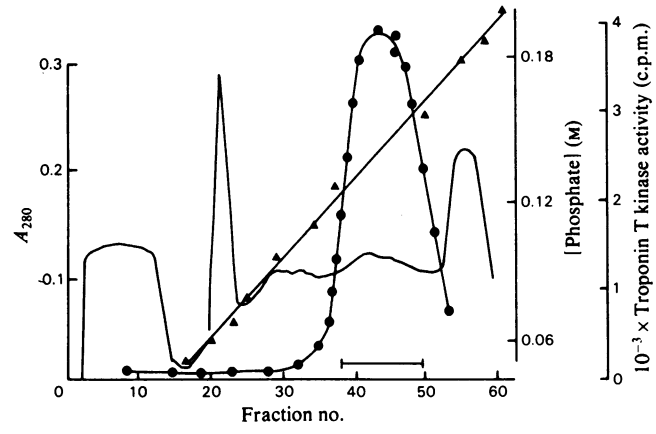

Fig. 2. *Chromatography of troponin T kinase on hydroxyapatite*
The dialysed fraction from the DEAE-cellulose column (Fig. 1) was applied to a column (2.2 cm × 11 cm) of hydroxyapatite equilibrated against buffer A. After washing out of unadsorbed materials with buffer A, the column was eluted with a linear phosphate gradient (50–250 mM) in a total volume of 480 ml. All solutions used contained 2 mM-EDTA and 2 mM-2-mercaptoethanol. The column was eluted at a flow rate of 40 ml/h and fractions were collected every 15 min. Troponin T kinase was assayed as described in the legend to Fig. 1. Symbols are as in Fig. 1.

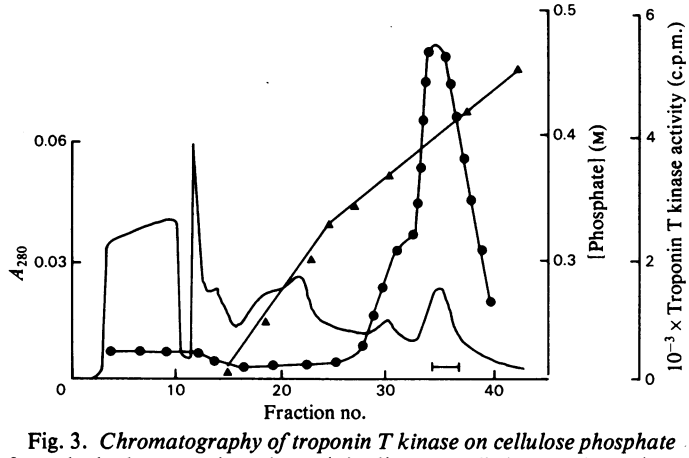

Fig. 3. *Chromatography of troponin T kinase on cellulose phosphate*
The dialysed fraction from the hydroxyapatite column (Fig. 2) was applied to a column (1.2 cm × 11 cm) of cellulose phosphate equilibrated against buffer A. After washing out of unadsorbed materials with buffer A, the column was eluted successively with 0.2 M-potassium phosphate, pH 7.0 (1 bed vol.), and with a linear phosphate gradient (0.2–0.5 M) in a total volume of 100 ml. All the solutions used contained 2 mM-EDTA and 2 mM-2-mercaptoethanol. The column was eluted at a flow rate of 14 ml/h and fractions were collected every 13 min. Troponin T kinase was assayed as described in the legend to Fig. 1. Symbols are as in Fig. 1.

increased up to 0.15 M. A large amount of contaminating proteins was eluted from the column immediately after troponin T kinase. The fractions possessing the troponin T kinase activity were pooled and dialysed against 3 vol. of buffer B for 3 h with vigorous stirring.

Further purification of troponin T kinase was achieved by chromatography on a cellulose phosphate column (Fig. 3). Troponin T kinase was strongly adsorbed by cellulose phosphate equilibrated against buffer A and was eluted only when the concentration of phosphate in the elution buffer was increased to 0.4 M. At this stage of purification the peak of the enzyme activity coincided well with the peak of the most tightly bound protein. The fractions of this peak were pooled and, after dialysis against 7 vol. of buffer B for 3 h with vigorous stirring, were applied to a column of immobilized dephosphorylated troponin T (Fig. 4). The single protein peak eluted from the column at about

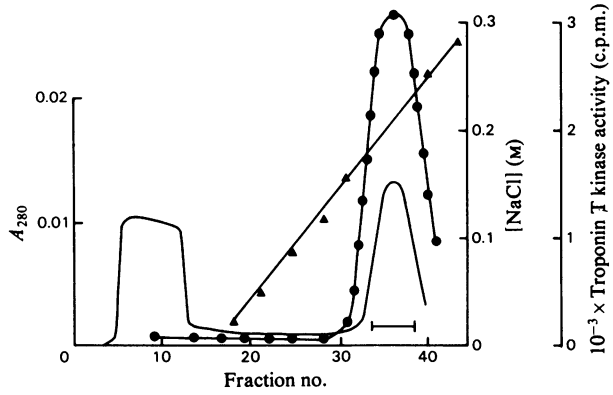

Fig. 4. *Chromatography of troponin T kinase on dephosphorylated troponin T, immobilized on Sepharose 4B*
The dialysed fraction from the cellulose phosphate column (Fig. 3) was applied to a column (0.9 cm × 15 cm) of troponin T–Sepharose 4B equilibrated with buffer A. After the column was washed with 1 bed vol. of buffer A, adsorbed material was eluted with a 80 ml linear gradient of 0–0.4 M-NaCl in 0.05 M-potassium phosphate, pH 7.0. All solutions used contained 2 mM-EDTA and 2 mM-2-mercaptoethanol. Fractions were collected every 20 min at a flow rate of 8 ml/h. Troponin T kinase was assayed as described in the legend to Fig. 1. Symbols are as in Fig. 1.

0.2 M-NaCl possessed a high protein kinase activity when troponin T was used as a substrate.

Unfortunately, at early steps of purification we failed to measure correctly the activity of troponin T kinase. Probably this failure was due to the presence of proteinases, to which troponin T is very susceptible, and/or of phosphatases in the crude preparations of protein kinase. The activity of troponin T kinase determined in the supernatant obtained after ultracentrifugation was about 0.06–0.1 nmol·min^{-1}·mg^{-1} of protein. The specific activity of the fraction eluted from the column of troponin T–Sepharose 4B reached 400–800 nmol·min^{-1}·mg^{-1} of protein.

Thus, in the different steps of purification, beginning with the DEAE-cellulose chromatography and up to affinity chromatography, 7000–10000-fold purification was achieved. The yield of the enzyme was 0.05–0.10 mg/kg of mixed rabbit skeletal muscles (eight preparations).

Molecular weight and protein composition of troponin T kinase

For the molecular-weight determination of native troponin T kinase the fraction eluted from the column of troponin T–Sepharose 4B was applied to a column (1.2 cm × 100 cm) of Sephadex G-200 equilibrated against 100 mM-potassium phosphate (pH 7.0)/2 mM-2-mercaptoethanol. The troponin T kinase activity and the protein were eluted from the column in a single symmetrical peak, which appeared in the void volume of the column. The elution pattern was essentially the same when 0.01 or 1 mg of the enzyme was applied to the column. This result indicates that the molecular weight of troponin T kinase may exceed 500000; also aggregation and the asymmetric form of the protein can interfere with the exact molecular-weight determination under the conditions used.

On electrophoresis at pH 8.6 in 25 mM-Tris/160 mM-glycine in 5% polyacrylamide gel, the mobility of troponin T kinase was extremely low, though the isoelectric point of the enzyme, determined by electrofocusing in polyacrylamide gel, was about 7.1. This fact may be indirect evidence for a high molecular weight of troponin T kinase, or strong aggregation of the enzyme.

The protein composition determined by electrophoresis in polyacrylamide gel in the presence of sodium dodecyl sulphate was slightly different for different troponin T kinase preparations (see gels *a–d* in Fig. 5). Of all the protein bands, three proteins with apparent mol.wts. 31000, 46000 and 50000 were most prominent and were consistently found in all troponin T kinase preparations. The components with mol.wts. of 50000 and 46000 are poorly resolved in the electrophoresis system of Weber & Osborn (1969). Better separation of these components was achieved in the discontinuous buffer system that had been used previously for investigation of the protein composition of the rabbit myofibrils (Schelud'ko, 1974).

All four preparations of troponin T kinase, the electrophoretograms of which are shown in Fig. 5, had approximately the same protein kinase activity, 400–800 nmol·min^{-1}·mg^{-1} of protein. Introduction of additional purification steps (e.g. rechromatography on hydroxyapatite or immobilized troponin T and chromatography on 5'-AMP–Sepharose) did not affect the composition of protein kinase.

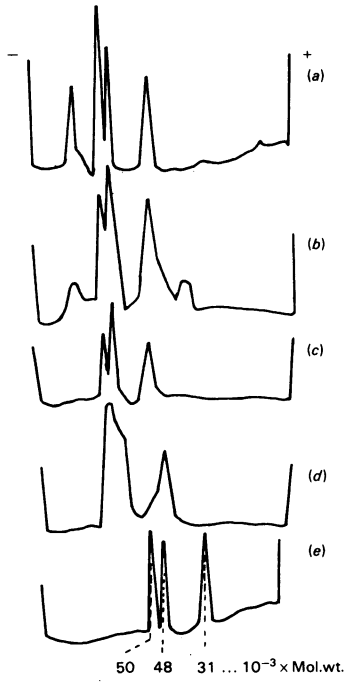

Fig. 5. *Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of troponin T kinase*

Curves (*a*)–(*d*) represent gel scans of four different preparations of troponin T kinase run in the system of Weber & Osborn (1969). Curve (*e*) represents the same preparation of the enzyme as in gel (*d*), but run in the system of Schelud'ko (1974). After reduction by an excess of 2-mercaptoethanol in the presence of 8 M-urea, 5–15 μg of enzyme was loaded on each gel.

These facts may suggest that the above-mentioned proteins, which were consistently present in all preparations, are the components of troponin T kinase.

Properties of troponin T kinase

Troponin T kinase is dependent on Mg^{2+} ions for activity. Ca^{2+}, cyclic AMP or cyclic GMP have no effect on the rate of troponin T phosphorylation by the highly purified enzyme as well as by the preparation of the enzyme obtained after DEAE-cellulose chromatography.

The pH–activity profile of the troponin T kinase is rather broad, with optimum in the range pH 7.0–7.5. At pH 5.7 and 8.6 there was 75% of maximum activity.

The reaction of troponin T phosphorylation by the isolated enzyme followed Michaelis–Menten kinetics in the range of ATP concentrations studied, from 2 to 50 μM. The apparent K_m for ATP determined from a double-reciprocal plot was 8.5 ± 0.5 μM.

Table 1. *Substrate specificity of troponin T kinase*

The values of the initial phosphorylation rates of different proteins are shown as percentages of the rate of troponin T phosphorylation. Concentration of all proteins was 0.25 mg/ml.

Substrate	Relative activity (%)
Troponin T	100
Phosvitin	20
Casein	10
Albumin	3
Protamine	3
Histone H1	5
Histone H2a	3
Histone H2b	3
Histone H3	4
Histone H4	3
Phosphorylase *b*	3

With increasing KCl concentration above 60 mM in the assay medium, the activity of troponin T kinase decreases and reaches nearly zero as the concentration of KCl approaches 0.6 M.

Troponin T kinase is highly specific for troponin T. No other substrate (see Table 1) is phosphorylated at a rate more than 20% of that obtained with the dephosphorylated troponin T. Within the reconstituted troponin complex, only troponin T is phosphorylated by the isolated enzyme.

The data from Table 1 suggest that, apart from troponin T, acid proteins (casein and phosvitin) are preferentially phosphorylated by troponin T kinase. This fact is rather unexpected, because the isoelectric point of troponin T is around 8.0 (Wilkinson, 1974). The unusual substrate specificity and a very high rate of troponin T phosphorylation raise the question of the phosphorylation site in the troponin T structure.

Determination of the site in troponin T phosphorylated by troponin T kinase

During long-term incubation with excesses of troponin T kinase, 0.85–0.95 mol of phosphate/mol of dephosphorylated troponin T is incorporated. This may indicate that only one site in the troponin T structure is phosphorylated by the isolated kinase. Previously we have reported a determination of the site of troponin T phosphorylated by a partially purified preparation of troponin T kinase (Gusev *et al.*, 1978). We reinvestigated this problem by using enzyme preparations of higher purity. When a tryptic digest of ^{32}P-labelled troponin T was applied to a column of Bio-Gel P-4, which was eluted by 0.1 M-HCl, all the radioactivity was eluted with the first well-separable peak, which absorbed at 206 nm but not at 280 nm (Fig. 6). This suggests that the site

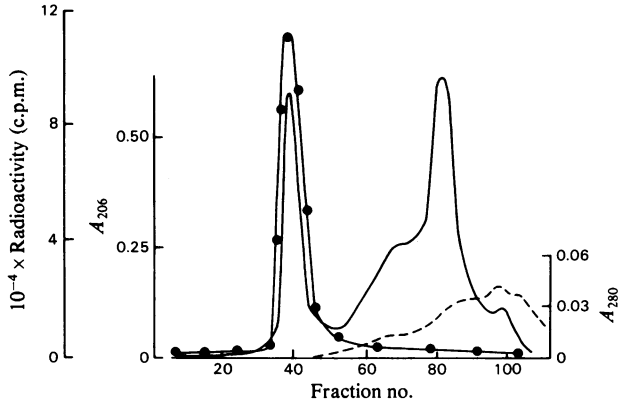

Fig. 6. *Chromatography of a tryptic hydrolysate of ^{32}P-labelled troponin T on Bio-Gel P-4*

A tryptic digest of [^{32}P]phosphorylated troponin T (approx. 4mg) was applied to a column (1.2cm × 100cm) of Bio-Gel P-4 equilibrated against 0.1M-HCl. The column was eluted at a flow rate of 4ml/h and 1ml fractions were collected. ——, A_{206}; – – – –, A_{280}; ●, radioactivity measured by the Čerenkov method.

of phosphorylation is localized in the largest *N*-terminal peptide, containing 40 amino acid residues (Moir *et al.*, 1977). Further hydrolysis of this peptide by Pronase and chromatography of the resulting mixture on a Dowex 50 (X2) column (see the Materials and Methods section) gave a radioactive peptide having the composition $Asp_1Ser_1Glu_3Val_1$. This peptide is identical with the *N*-terminal hexapeptide of troponin T (Moir *et al.*, 1977; Pearlstone *et al.*, 1976). Thus the enzyme isolated phosphorylates only the *N*-terminal serine residue, i.e. the site in troponin T that is normally phosphorylated in the whole troponin complex isolated from skeletal muscles.

Discussion

Using the method described here, we have isolated an enzyme exhibiting some unusual properties. In its strong adsorption by DEAE-cellulose and hydroxyapatite and high sensitivity to the increase in ionic strength, the enzyme under study is similar to cyclic AMP-dependent protein kinase (Huang & Huang, 1975; Walsh *et al.*, 1968). However, the troponin T kinase is strongly adsorbed by cellulose phosphate and phosphorylates acid proteins (casein and phosvitin) at a rate exceeding that for basic proteins (histones and protamine). These properties of the isolated enzyme are similar to those of protein kinases phosphorylating acid (nuclear) proteins (Dahmus, 1976; Goldstein & Hasty, 1973). Finally, the high molecular weight and protein composition of troponin T kinase differ from the corresponding properties of both cyclic AMP-dependent and nuclear protein kinases.

The primary structure of the site phosphorylated by troponin T kinase, i.e. AcSer-Asp-Glu-Glu-Val-Glu- (Ac = acetyl) (Pearlstone *et al.*, 1976), is very peculiar. The sites phosphorylated by the cyclic AMP-dependent protein kinase are characterized by the following: the residue of basic amino acid is localized at a distance of one or two amino acid residues from the phosphorylated serine (Williams, 1976; Daile *et al.*, 1975). However, the first basic amino acid residue (lysine) in the troponin T primary structure is separated from the *N*-terminal serine residue by 40 amino acids. At the same time the structure of the troponin T phosphorylation site is similar to the structure of sites phosphorylated in the acidic proteins phosvitin and casein, where the residue of glutamic acid or phosphoserine is localized at a distance of two amino acid residues from the phosphorylated serine residue (Mercier *et al.*, 1971; Mackinlay *et al.*, 1977). This similarity in the primary structure may be a possible explanation for phosvitin and casein phosphorylation by troponin T kinase. It should be noted that an essential role in the recognition of a site of troponin T phosphorylation belongs to the secondary and tertiary structure of the protein. Evidence for this assumption may be derived from the fact that in a mixture of troponin T tryptic peptides protein kinase is unable to phosphorylate the *N*-terminal acetylated serine residue.

There exist four enzymes that are known to phosphorylate isolated troponin T or troponin T in the whole troponin complex, i.e. phosphorylase *b* kinase (Moir *et al.*, 1977), an enzyme that is similar to phosphorylase *b* kinase and is tightly bound to the sarcoplasmic reticulum (Varsanyi *et al.*, 1978), cyclic AMP-dependent protein kinase (Pratje & Heilmeyer, 1972; Korovkin & Antipenko, 1979) and

troponin T kinase described in the present paper. According to the general viewpoint, neither phosphorylase *b* kinase nor cyclic AMP-dependent protein kinase nor a reticulum-bound kinase are able to phosphorylate troponin T *in vivo*. Troponin T kinase possesses a high specific activity and substrate specificity and can only phosphorylate the *N*-terminal serine residue, i.e. the site that is phosphorylated when troponin T is isolated from skeletal muscles. Therefore we may assume that the enzyme isolated phosphorylates troponin T *in vivo*.

Kumon & Villar-Palasi (1979) have reported the isolation of troponin T kinase. Apparently we are dealing with the same enzyme, although our method provides higher purification. The absence of influence of Ca^{2+}, cyclic AMP and cyclic GMP on the enzyme activity and the similar substrate specificity prove this suggestion. These enzymes are able to phosphorylate only acid proteins, i.e. phosvitin and casein, beside troponin T.

Some difference in properties (pH optimum, K_m for ATP and substrate specificity) can be explained by various conditions and/or different enzyme purity.

The protein composition of troponin T kinase, the regulatory mechanisms of enzyme activity and the physiological role of phosphorylation require further investigation.

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