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Phosphorylation of Troponin and the Effects of Interactions Between the Components of the Complex

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1. The troponin complex from skeletal muscle contains approximately 1 mol of phosphate/80000g of complex, covalently bound to the troponin T component. 2. On prolonged incubation of the troponin complex or troponin T with phosphorylase kinase the phosphate content of troponin T was increased to approx. 3mol/mol. 3. On prolonged incubation of troponin I with phosphorylase kinase up to 1.6mol of phosphate/mol were incorporated. 4. Phosphorylation of troponin I was greatly inhibited by troponin C owing to the strong interaction between these proteins. Thus in the troponin complex troponin T was the main substrate for phosphorylase kinase. The phosphorylation of isolated troponin T was also inhibited by troponin C. 5. Troponin I was phosphorylated when the troponin complex was incubated with a bovine cardiac 3′:5′-cyclic AMP-dependent protein kinase. Troponin T either in its isolated form or in the troponin complex was not phosphorylated by bovine protein kinase to any significant extent under the conditions used. 6. If the troponin complex was dephosphorylated to 0.2mol/mol, or phosphorylated up to 2.5mol/mol there was no significant effect on the ability of normal concentrations to confer Ca^{2+} sensitivity on the adenosine triphosphatase of densensitized actomyosin.

The protein system in the myofibril involved in the regulation of contractile activity by Ca^{2+} consists of tropomyosin and the troponin complex. It is now well established that the latter complex consists of troponin C, the calcium-binding protein, troponin I, the protein that inhibits the Ca^{2+}-stimulated ATPase* of actomyosin and troponin T (for reviews see Perry, 1973; Weber & Murray, 1973). The function of troponin T is less well defined but there is evidence that it will interact with tropomyosin (Greaser & Gergely, 1971) and troponin C (Ebashi *et al.*, 1972; Van Eerd & Kawasaki, 1973). The original report on phosphorylation of the troponin complex by Bailey & Villar-Palasi (1971) using a 3′:5′-cyclic AMP-dependent protein kinase from muscle has raised the question whether phosphorylation is also involved in the regulation of contractile activity. Several other groups of workers have reported phosphorylation of the troponin complex by phosphorylase kinase and a 3′:5′-cyclic AMP-dependent protein kinase, both isolated from skeletal muscle (Stull *et al.*, 1972; Pratje & Heilmeyer, 1972; Perry & Cole, 1973), but there is no agreement as to which of the components of the troponin complex are phosphorylated and the specificities of the enzymes involved. For example, whereas Bailey & Villar-Palasi (1971) obtained phosphorylation of troponin I, Pratje & Heilmeyer (1972) reported that troponin T was the principal acceptor with a 3′:5′-cyclic AMP-dependent protein kinase. Likewise we have reported (Perry & Cole 1973) troponin T as the principal acceptor when phosphorylase kinase is used to phosphorylate troponin, but Stull *et al.* (1972) found troponin I to be the main acceptor when troponin B (Hartshorne & Mueller, 1968) was used as substrate for this enzyme.

The present work is an extension of our earlier work and demonstrates that interaction with troponin C can inhibit the phosphorylation of troponin I and troponin T. Thus the phosphorylase kinase-catalysed phosphorylation of troponin I that occurs readily with the protein alone or when it is associated with troponin T, is much decreased when it is present as the troponin complex. Some of the findings have been briefly reported elsewhere (Perry & Cole, 1974).

* Abbreviation: ATPase, adenosine triphosphatase.

Methods and Materials

Methods

Separation of the components of troponin. Troponin was prepared from the back and leg muscles of the rabbit, the breast and leg muscles of the chicken and the back and legs of the mouse by the method of Ebashi *et al.* (1971) and from bovine and rabbit heart muscles as described by Tsukui & Ebashi (1973). It was separated into its components on DEAE-cellulose by a modification of the method of Greaser & Gergely (1971), and when necessary troponin I

and troponin T were rechromatographed on CM-cellulose by a method similar to that of Wilkinson (1974).

DEAE-cellulose DE52 (Whatman Biochemicals Ltd., Maidstone, Kent, U.K.) was washed once with water and equilibrated with 8M-urea–15mM-2-mercaptoethanol–50mM-Tris–HCl buffer (pH8.0) at room temperature. Urea for all chromatographic separations was de-ionized as a 9M solution on a Biodeminrolit (British Drug Houses Ltd., Poole, Dorset, U.K.) mixed-bed ion-exchange resin immediately before use. Troponin solution (100–200ml; 10mg/ml) was dialysed against three changes of starting buffer and applied under gravity to a column (3cm×20cm). Buffer was then pumped on to the column at a rate of 50ml/h. Troponin I was eluted with the starting buffer and application of a gradient of 0–0.3M-NaCl in the above buffer eluted two peaks corresponding to troponin T and troponin C, in that order. The final peak of troponin C was well-separated and consisted of virtually pure material. On electrophoresis on 10% (w/v) polyacrylamide gels in 0.1% sodium dodecyl sulphate–0.04M-monobasic sodium phosphate–0.06M-dibasic sodium phosphate buffer (pH7.0), troponin C migrated as a single band when up to 0.1mg was applied to the gel. By selecting fractions from the other peaks, samples of troponin I and troponin T of similar purity to the troponin C could be obtained (Plate 1, Fig. 1). Less-pure samples of these proteins were applied to columns of CM52 cellulose (Whatman Biochemicals Ltd.) equilibrated with 8M-urea–15mM-2-mercaptoethanol–0.1M-sodium acetate–0.15M-acetic acid, pH 4.5. Any troponin C was eluted with the starting buffer and troponin I and troponin T were eluted in that order by application of a gradient of 0–0.2M-NaCl in the buffer.

Material from the peaks was pooled and dialysed against distilled water in the case of troponin C and 10mM-HCl for fractions containing troponin T and troponin I, since these proteins were insoluble in water. After five changes of dialysis fluid the fractions were freeze-dried and stored at −20°C.

Tropomyosin. This protein was prepared by the method of Cummins & Perry (1973).

Assay of actomyosin ATPase. ATPase assays were carried out as described by Schaub & Perry (1969) by using desensitized actomyosin prepared by the modified method of Cummins & Perry (1973).

Phosphorylase kinase experiments. In most of the experiments crude phosphorylase kinase, prepared as described by Krebs *et al.* (1964), was further purified by chromatography on Sepharose 4B (Pharmacia Fine Chemicals AB, Uppsala, Sweden) as described by Brostrom *et al.* (1971) or by Cohen (1973) (see below). The enzyme (0.05–0.2mg/ml) was incubated at 30°C with the troponin complex or components derived from it (0.5–2mg/ml) in 20mM-Tris–20mM-sodium glycerophosphate buffer (pH8.6) containing 10mM-magnesium acetate, 0.5mM-$CaCl_2$, 25mM-NaF, 1mM-dithiothreitol and 0.5–2.5mM-NaATP (pH7.0), which in many experiments contained [γ-^{32}P]ATP (The Radiochemical Centre, Amersham, Bucks., U.K.) of known specific radioactivity. With incubations containing troponin I or troponin T the incubation medium contained 50mM-KCl to aid solubilization in addition to these components. Troponin was dissolved in 2mM-$NaHCO_3$; troponin C in 50mM-Tris–HCl (pH8.0); troponin I in 1mM-HCl–0.5M-KCl and troponin T in 10mM-HCl–0.5M-KCl. The solutions of troponin I and troponin T were saturated with pepstatin (Banyu Pharmaceutical Co. Ltd., Tokyo, Japan) and diluted fivefold in the incubation medium, which was then adjusted to pH8.2 with 25mM-Tris. Samples (1–2ml) were withdrawn after known times of incubation and precipitated with 0.5vol. of ice-cold 15% (w/v) trichloroacetic acid. The controls contained all additions except troponin or components which were added after the trichloroacetic acid so that the final amount of protein was the same in all cases. The precipitated protein was diluted with an equal volume of 5% (w/v) trichloroacetic acid containing 2.5mM-ATP, centrifuged for 5min at 2000rev./min in a Mistral 2L centrifuge at 5°C and washed three times with ice-cold 5% trichloroacetic acid by resuspension and centrifugation. Total phosphate analyses and determinations of radioactivity were carried out on the washed protein. Phosphorylase kinase activity was determined by the method of Cohen (1973).

Determination of phosphate. Phosphorus covalently bound to protein that had been repeatedly washed with trichloroacetic acid as described above (see under 'Phosphorylase kinase experiments') was determined in two ways. (1) By ^{32}P determination in which the protein was dissolved in 0.5ml of 0.5M-NaOH and diluted with up to 10ml of distilled water. The radioactivity was measured (see below) and samples were taken for determination of total protein by the method of Lowry *et al.* (1951) with bovine serum albumin as standard. From the specific radioactivities of the [γ-^{32}P]ATP and the phosphorylated protein the amount of phosphate transferred to the protein was calculated.

(2) By direct total phosphorus determination by the method of Bartlett (1959). In some cases when [γ-^{32}P]ATP was used the radioactivity of the digest was also determined before the addition of ammonium molybdate. Protein determinations were done by a method involving Nesslerization after digestion (Strauch, 1965), by assuming that the protein contained 16% N.

Determination of radioactivity. ^{32}P was determined by the Čerenkov method (Gould *et al.*, 1972) at an efficiency of 25 or 33% by using a Philips model PW4510 liquid-scintillation analyser. Clear colourless

solutions of trichloroacetic acid-precipitated protein in 0.025M-NaOH, protein digests in 1M-H_2SO_4, and slices of polyacrylamide gels containing protein bands that had been digested in H_2O_2, were prepared for these determinations.

Separation of troponin components by electrophoresis. For the determination of ^{32}P incorporation into the components of troponin, trichloroacetic acid-precipitated proteins were dissolved and dialysed against 4% (w/v) sodium dodecyl sulphate–5M-urea–1% (w/v) 2-mercaptoethanol–10mM-sodium phosphate buffer (pH7.0) at 37°C overnight before their electrophoresis on 10% (w/v) polyacrylamide gels in 0.04M-monobasic sodium phosphate–0.06M-dibasic sodium phosphate (pH7.0)–0.1% sodium dodecyl sulphate buffer (Weber & Osborn, 1969). In the experiments involving formation of the complex of troponin C with troponin I, electrophoretic separation was carried out on 10% (w/v) polyacrylamide gels in 8M-urea–20mM-Tris–125mM-glycine (pH8.3) (Perrie *et al.*, 1973). After the phosphorylation of mixtures of the proteins the reaction was stopped by the addition of solid urea to 5M concentration and the solution applied directly to the urea gels.

The gels were stained with Coomassie Brilliant Blue 'R' (Edward Gurr Ltd., High Wycombe, Bucks., U.K.) and slices containing single protein bands were cut out, digested in 2ml of H_2O_2 (100vol.) in stoppered vials at 80°C for 3h and diluted to 7ml for ^{32}P determination. The ratio of the amounts of the components was found by densitometric scanning of the gels in a Chromoscan J312 (Joyce Loebl Co., Gateshead-on-Tyne, U.K.).

Dephosphorylation of the troponin complex. Troponin (5mg/ml) was dissolved in 0.5M-Tris–HCl buffer (pH8.0) and incubated with *Escherichia coli* alkaline phosphatase (Worthington, Cambrian Chemicals Ltd., Beddington Farm Road, Croydon, Surrey, U.K.) at 0.2mg/ml at 30°C for 1h, i.e. optimum conditions for *E. coli* alkaline phosphatase (Garen & Levinthal, 1960). The same amount of enzyme was added, the incubation continued for a further 2–4h and the mixture dialysed against 10mM-Tris–HCl buffer (pH7.5) before use.

Materials

Bovine cardiac 3′:5′-cyclic AMP-dependent protein kinase and bovine serum albumin used as a standard for protein estimations were purchased from Sigma (London) Chemical Co. Ltd., Kingston-upon-Thames, Surrey, U.K.

Results

Preparation of troponin

In our laboratory chromatography on DEAE-cellulose in 8M-urea gave a better separation of the troponin constituents than did chromatography on DEAE-Sephadex in 6M-urea (Greaser & Gergely, 1971). Good separation was obtained on DEAE-cellulose, if the original troponin preparation was fairly free from contaminants such as tropomyosin and proteolytic breakdown products (Fig. 1, Plate 1). Proteolysis was minimized during the preparation by working rapidly at low temperatures when possible and by including the cathepsin D inhibitor, pepstatin (Barrett & Dingle, 1972) during the LiCl-extraction procedure. Usually only traces of tropomyosin were present in the troponin preparation, and these were eluted with other impurities as a small peak soon after application of the gradient. In some preparations partial proteolysis of troponin T produced components of about 28000 and 22000 daltons which were eluted as shoulders on the trailing edge of the protein peak containing troponin T (Fig. 1).

No steps were taken to exclude traces of Ca^{2+} during the separation and so troponin I and troponin C would be present as a complex (Perry *et al.*, 1972; Head & Perry, 1974). The separation achieved in the presence of the cation exchanger implies that troponin C binds to DEAE-cellulose rather than to troponin I under these conditions although troponin C and troponin I migrate as the complex on polyacrylamide-gel electrophoresis at a similar pH value and urea concentration (Perry *et al.*, 1972). With the anion exchangers SE-Sephadex (Schaub *et al.*, 1972) and SP-Sephadex (Head & Perry, 1974) at pH6.0–6.5 in 6–8M-urea, troponin I and troponin C are eluted as the complex. This can be dissociated by including EGTA [ethanedioxybis(ethylamine)tetra-acetic acid] in the elution buffer. During chromatography on CM-cellulose, however, in 8M-urea at pH4.5 (Wilkinson, 1974) the complex is dissociated and good separation can be obtained without adding EGTA to the buffer. This procedure was used to further purify troponin I and troponin T fractions after preliminary separation on DEAE-cellulose. If there was significant cathepsin D contamination, however, some proteolysis occurred during chromatography under these conditions.

Phosphorylation of troponin complex by phosphorylase kinase

Our earlier experiments (Perry & Cole, 1973) on the phosphorylation of the troponin complex were carried out with a relatively crude phosphorylase kinase preparation and it was considered that this might account for the different pattern of ^{32}P incorporation into the components of the complex compared with that reported by Stull *et al.* (1972). Phosphorylase kinase prepared from rabbit skeletal muscle by the method of Krebs *et al.* (1964) was therefore further purified by gel filtration on Sepharose 4B as described by Brostrom *et al.* (1971)

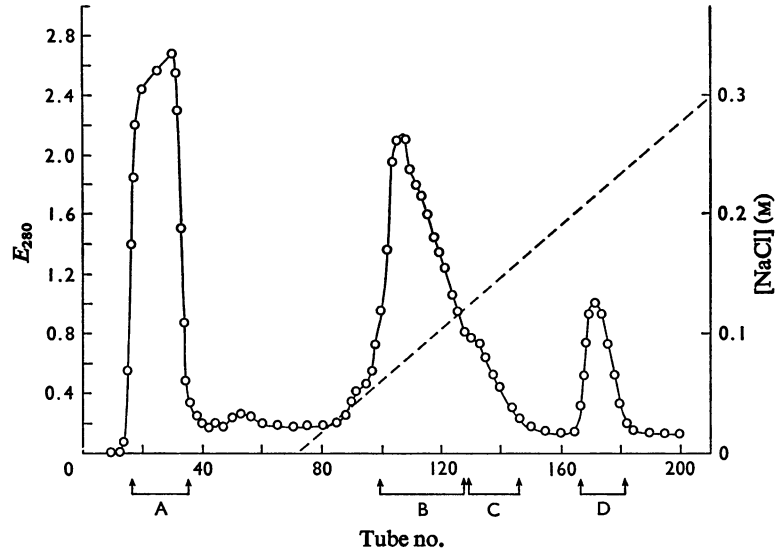


Fig. 1. *Fractionation of the troponin complex by chromatography on DEAE-cellulose*

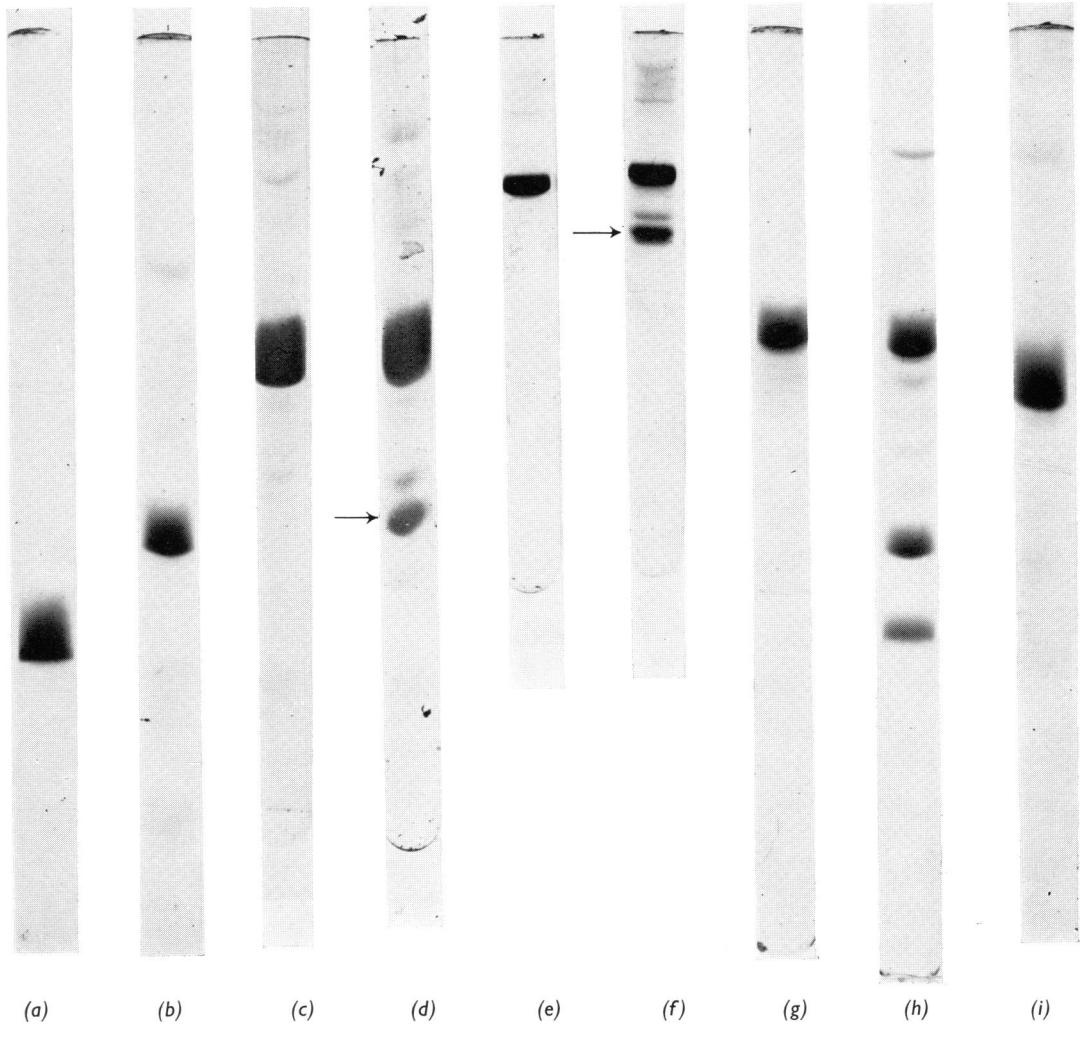
A solution (80ml) (E_{280} 13.7) of troponin complex isolated from rabbit skeletal muscle, dialysed against 8M-urea–15mM-2-mercaptoethanol–50mM-Tris–HCl buffer (pH8.0) at room temperature was applied to a column (3cm×22cm) of DEAE-cellulose (DE52) equilibrated with the same buffer. A linear gradient to 0.3M-NaCl in the starting buffer was applied at a rate of 50ml/h and 5ml fractions were collected. Fractions A, B and D contained troponin I, troponin T and troponin C respectively. In fraction C proteolytic fragments of molecular weight 30000–22000 and lower molecular weights derived from troponin T were eluted if present (see Plate 1*d* and 1*f*). ----, NaCl concn.

or by Cohen (1973). Three main protein peaks were obtained by this procedure. Significant incorporation of ^{32}P into whole troponin was only obtained on incubation with a portion of the second of the three peaks, which mainly consisted of phosphorylase kinase. Unless otherwise stated all experiments reported here on the phosphorylation of troponin and its components were carried out with phosphorylase kinase purified in this way.

The third peak eluted was rich in phosphorylase but also contained traces of an enzyme provisionally designated 'myosin light-chain kinase', which phosphorylates the 18000 dalton light-chain component of myosin from rabbit skeletal muscle (Pires, *et al.*, 1974; see Perrie *et al.*, 1973). Fractions from the third peak or more purified preparations of 'myosin light-chain kinase' did not phosphorylate troponin at a significant rate.

Troponin preparations from rabbit and fowl skeletal muscles were incubated with [γ-^{32}P]ATP and phosphorylase kinase, subsequently fractionated by electrophoresis on polyacrylamide gels in sodium dodecyl sulphate and the radioactivity was determined in slices containing single bands of protein. With troponin from mixed skeletal muscle of the rabbit, irrespective of whether incubation was carried out with purified or crude preparations of phosphorylase kinase, 75–80% of the total radioactivity was associated with the troponin T band, corresponding to a molecular weight of 37000 (Table 1). Significant radioactivity was also present in the band corresponding to troponin I but not in that corresponding to troponin C. In the case of the troponin complex from mixed skeletal muscle of the fowl, in addition to protein bands of 18000 and 23000 daltons, corresponding to troponin C and troponin I respectively, bands of 37000 and 44000 daltons were also obtained (Table 1, Plate 1). Most of the phosphorylation was associated with these two bands, that corresponding to 44000 daltons invariably showing the highest radioactivity. Densitometric scanning of stained gels showed that there was always more of the 44000 dalton component than the 37000 dalton component in these preparations and the specific radioactivity of the two components after incubation with [γ-^{32}P]ATP and phosphorylase kinase was approximately the same.

When troponin was prepared from chicken breast muscle it consisted principally of troponin C, troponin I and a 44000 dalton component (Hitchcock, 1973). If troponin was prepared from leg muscle the 44000 dalton band was replaced by a band of 37000 daltons. Thus it appeared that the 44000 and 37000 dalton components were characteristic of breast and



EXPLANATION OF PLATE 1

Polyacrylamide-gel electrophoresis of components of the troponin complex

Except for the gels illustrated in (*e*) and (*f*) electrophoresis was carried out in 10% (w/v) polyacrylamide, 0.1% sodium dodecyl sulphate, 100mM-sodium phosphate buffer (pH7.0). Approximately 80μg of protein was applied unless otherwise stated. (*a*) Troponin C from rabbit mixed skeletal muscle. (*b*) Troponin I from rabbit mixed skeletal muscle. (*c*) Troponin T from rabbit mixed skeletal muscle; fraction B, Fig. 1. (*d*) Troponin T with 22000 dalton proteolytic fragment (marked with arrow); fraction C, Fig. 1. (*e*) Troponin T as in (*c*) run in 6M-urea–5% (v/v) acetic acid (pH3.2); 10μg of protein in 8M-urea was applied to the gel. (*f*) Troponin T as in (*d*) run in 6M-urea–5% (v/v) acetic acid (pH3.2); 10μg of protein was applied as in (*e*). The 22000 dalton component is indicated by an arrow. (*g*) Troponin T, 44000 dalton component, from chicken breast muscle. (*h*) Troponin complex from chicken breast muscle. (*i*) Troponin T, 37000 dalton component, isolated from chicken leg muscle.

Table 1. *Incorporation of ^{32}P into troponin incubated with [γ-^{32}P]ATP and phosphorylase kinase preparations isolated from mixed skeletal muscle of rabbit*

For incubation conditions see the Methods and Materials section. Values in parentheses represent number of different enzyme preparations used. Molecular weights of troponin components are indicated at the top of the columns. Values are percentages, the band with the highest radioactivity being taken as 100% in each case.

Source of troponin	No. of experiments	Method of phosphorylase preparation	Incorporation of ^{32}P into components (%)			
			Troponin T (44000)	(37000)	Troponin I (23000)	Troponin C (18000)
Rabbit mixed skeletal muscle	4 (2)	Krebs *et al.* (1964)		100	17	5
	5 (4)	Brostrom *et al.* (1971)		100	12	3
	3 (2)	Cohen (1973)		100	14	3
Chicken mixed skeletal muscle	3 (2)	Brostrom *et al.* (1971)	100	58	8	3

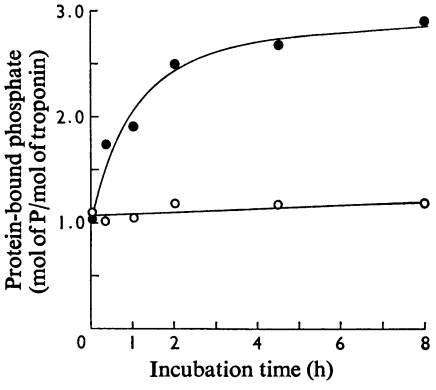


Fig. 2. *Phosphorylation of the troponin complex of rabbit skeletal muscle by phosphorylase kinase*

Troponin (1 mg/ml) was incubated with phosphorylase kinase (0.08 mg/ml) and 2.5 mM-ATP at pH 8.2 under the assay conditions described in the Methods and Materials section. Samples were withdrawn at the intervals shown and the protein-bound phosphate was determined. The molecular weight of the troponin complex was taken to be 80000. ●, Troponin incubated with enzyme; ○, control (enzyme incubated alone), troponin was added after trichloroacetic acid.

leg muscles respectively. Since they were both readily phosphorylated in troponin isolated from mixed chicken muscle they may represent polymorphic forms of troponin T corresponding to the different muscle types.

Troponin from rabbit skeletal muscle was phosphorylated rather slowly by the purified phosphorylase kinase. The rate was about 300 times lower than with phosphorylase *b* as substrate (assays kindly carried out by Dr. P. Cohen). Nevertheless, after incubation with the enzyme for several hours the total phosphate content, determined by direct estimation of protein-bound phosphorus, reached a maximum of about 3 mol of P/80000 g (Fig. 2). Since the troponin contained 1.0 mol of P/80000 g (Table 2) when isolated, this indicated that up to a maximum of an additional 2 mol of P was incorporated/mol of troponin. Similar results were obtained when the incorporation of phosphate was calculated from the specific radioactivity of the protein after incubation with [γ-^{32}P]ATP of known specific radioactivity. Troponin from chicken skeletal muscle also incorporated up to an additional 2 mol of P/mol when incubated with this phosphorylase kinase.

Phosphorylation of isolated components of troponin complex

Troponin T. Purified troponin T, which contained virtually all of the protein-bound phosphate present in the troponin complex isolated from rabbit mixed skeletal muscle (Table 2), incorporated an additional 2 mol of P/37000 g on prolonged incubation with phosphorylase kinase and ATP so that the fully phosphorylated protein contained a total of 3 mol of P/37000 g (Fig. 3). The total phosphate content of the 37000 and 44000 dalton components separately isolated from troponin from chicken leg and breast muscle respectively also approached 3 mol/mol in each case on prolonged incubation with phosphorylase kinase.

Troponin I. Whereas little phosphorylation of troponin I occurred in the troponin complex, compared with that obtained with troponin T, the isolated protein was phosphorylated by phosphorylase kinase at least as readily as purified troponin T. The rate was very much slower than with phosphorylase *b* as substrate (assays kindly carried out by Dr. P. Cohen) and on prolonged incubation up to 1.6 mol of P/mol of troponin I was incorporated as determined both by direct phosphorus analysis and by measurement of the specific radioactivity of the protein on incubation with [γ-^{32}P]ATP (Fig. 4).

Table 2. *Protein-bound phosphate in troponin preparations from skeletal and cardiac muscle*

Numbers of preparations analysed are in parentheses. Molecular weights are indicated at the head of each column.

Species	Muscle	Total phosphate (mol/mol) Troponin complex (80000)	Troponin C (18500)	Troponin I (23000)	Troponin T (37000)	Troponin T (44000)
Rabbit	Mixed skeletal	1.0 (7)	0.10 (3)	0.05 (4)	0.74 (9)	
	Cardiac	0.8–2.2 (3)				
Chicken	Breast	0.9 (1)	0.14 (1)	0.03 (1)		0.8 (1)
	Leg	0.8 (1)		0.04 (1)	0.8 (1)	
Mouse	Mixed skeletal	1.1 (2)				
Cow	Cardiac	0.7–1.7 (2)				

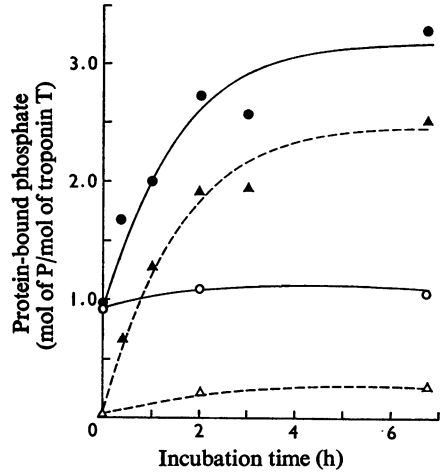


Fig. 3. *Phosphorylation of troponin T from rabbit skeletal muscle by phosphorylase kinase*

Troponin T (0.5 mg/ml) was incubated with phosphorylase kinase (0.08 mg/ml) and 2 mM-ATP containing [γ-^{32}P]-ATP at pH 8.2 under the conditions described in the Methods and Materials section. Samples were withdrawn at the intervals indicated and protein-bound total P and ^{32}P were determined. ●, Enzyme incubated with troponin T, direct determination of total phosphate; ○, control, troponin T added after trichloroacetic acid. ▲, Enzyme incubated with troponin T, phosphate incorporation determined from the specific radioactivity; △, control, troponin T added after trichloroacetic acid.

Phosphorylation catalysed by phosphorylase kinase and interactions between the components of the troponin complex and tropomyosin

The observation that troponin I was much more readily phosphorylated as the isolated protein than when present as the troponin complex suggested that interactions with other components of the complex might affect the accessibility of the phosphorylation sites.

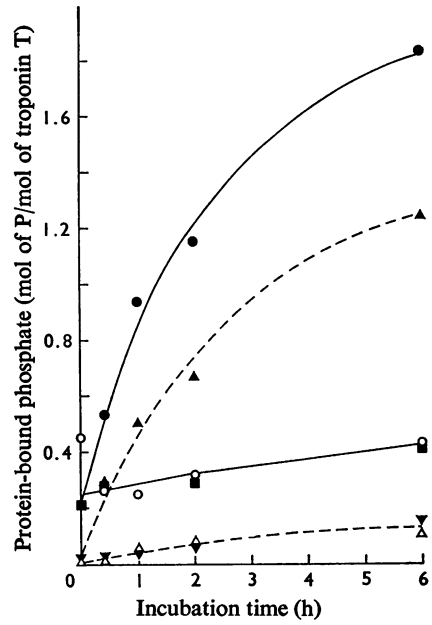


Fig. 4. *Effect of troponin C on the phosphorylation of troponin I*

Troponin I (0.8 mg/ml) from rabbit skeletal muscle was incubated with troponin C from the same source (0.8 mg/ml), phosphorylase kinase (0.11 mg/ml) and 2.5 mM-ATP containing [γ-^{32}P]ATP (0.5 μCi/ml) under the conditions described in the Methods and Materials section. ●, ▲, Enzyme incubated with troponin I, troponin C added after trichloroacetic acid; ○, △, enzyme incubated with troponin I and troponin C; ■, ▼, enzyme incubated, troponin I and troponin C added after trichloroacetic acid. ○, ●, ■, Protein-bound phosphate measured directly; ▲, △, ▼, protein-bound phosphate determined from the specific radioactivity.

When troponin T was mixed with troponin I phosphorylation of both components occurred, although after prolonged incubation of an equimolar

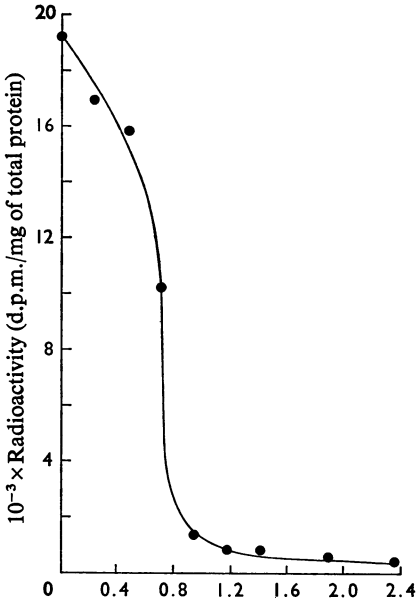


Fig. 5. *Effect of various amounts of troponin C on the phosphorylation of troponin I catalysed by phosphorylase kinase*

Troponin I (0.5mg/ml) from rabbit skeletal muscle was incubated with phosphorylase kinase (0.08mg/ml), 2.5mM-ATP containing [γ-^{32}P]ATP (1μCi/ml) and troponin C from rabbit skeletal muscle (0–10mg/ml) for 30min under the conditions described in the Methods and Materials section. After the incubation had been stopped with trichloroacetic acid, where necessary further amounts of troponin C were added so that the total amount was the same in each tube. The radioactivity incorporated into the enzyme, incubated under the same conditions without troponin components, has been deducted from the values plotted. Protein concentration was measured by Nesslerization throughout.

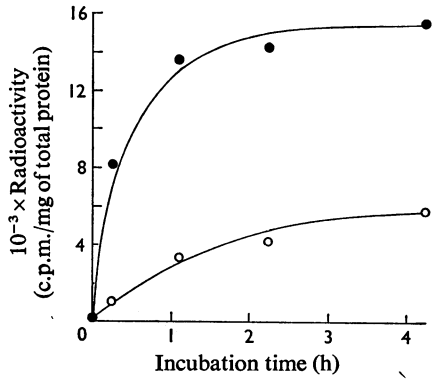


Fig. 6. *Effect of troponin C on the phosphorylation of troponin T*

Troponin T (0.25mg/ml) was incubated with phosphorylase kinase (0.07mg/ml) and 2.5mM-ATP containing [γ-^{32}P]ATP (1.0μCi/ml) in the presence and the absence of troponin C (0.2mg/ml) under the conditions described in the Methods and Materials section. After the addition of trichloroacetic acid, the protein concentration was adjusted to be the same in all tubes. The enzyme blank has been deducted from the values shown. ●, Troponin T incubated, troponin C added after trichloroacetic acid; ○, troponin T+troponin C incubated together.

mixture of the proteins, usually the troponin T contained more radioactive ^{32}P/mol (1.5–2.0 times greater than troponin I). When, however, troponin C was mixed with troponin I isolated from the same muscle, in molar ratios greater than 0.8:1, phosphorylation of troponin I was completely inhibited (Fig. 4). The extent of inhibition increased sharply as the molar ratio troponin C/troponin I rose from 0.6:1 to 0.8:1 (Fig. 5). Similar inhibition of phosphorylation by troponin C was obtained when the two components were derived from the skeletal muscle of different species such as the fowl and the rabbit. Phosphorylation of troponin I did not prevent complex formation with troponin C, for when troponin I phosphorylated with [γ-^{32}P]ATP was mixed with troponin C and electrophoresed in 8M-urea–20mM-Tris–125mM-glycine buffer (pH8.3) a radioactive band that migrated with a mobility corresponding to that of the equimolar complex of troponin I and troponin C (Perry *et al.*, 1972; Head & Perry, 1974) was obtained. When the sample was treated with EGTA before electrophoresis the band corresponding to the complex was replaced by a radioactive band at the origin (troponin I) and a fast-moving unlabelled band corresponding to troponin C.

Troponin C also inhibited the phosphorylation of troponin T by phosphorylase kinase although the inhibition was not as complete as with troponin I. When troponin C and troponin T were present in 1:1 molar ratio the incorporation of phosphorus by troponin T was about 30% of that obtained in the absence of troponin C (Fig. 6). The extent of inhibition increased gradually as the molar ratio troponin C/troponin T rose from zero to 1:1.

The inhibition of the phosphorylation of troponin I and troponin T by troponin C was not due to inhibition of phosphorylase kinase for no significant inhibition of the conversion of phosphorylase *b* into phosphorylase *a* could be demonstrated with troponin C and phosphorylase kinase in the weight ratio of 10:1.

Tropomyosin added in equimolar amounts did not significantly affect the rate of phosphorylation

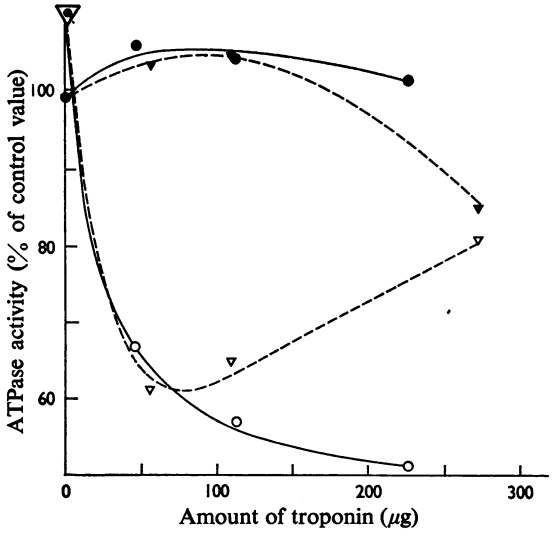


Fig. 7. *Effect of phosphorylation by phosphorylase kinase on the biological activity of troponin from rabbit skeletal muscle*

Troponin was phosphorylated with phosphorylase kinase and ATP as described in the Methods and Materials section. After 5h incubation when the total phosphate content was 2.5mol/80000g the incubation mixture was dialysed against 10mM-Tris–HCl (pH7.5). The unincubated control troponin was dialysed against the same buffer. The effect of increasing amounts of this troponin on the Mg^{2+}-activated ATPase of actomyosin was measured after incubation in a medium containing 300μg of desensitized actomyosin, 100μg of tropomyosin, 2.5mM-$MgCl_2$, 2.5mM-Tris–ATP, 25mM-Tris–HCl buffer (pH7.6) in the presence and absence of 1.25mM-EGTA after incubation for 5min at 25°C. ●, Control troponin; ○, control troponin +EGTA; ▼, phosphorylated troponin; ▽, phosphorylated troponin+EGTA.

of the troponin complex by phosphorylase kinase. When troponin T was incubated with tropomyosin under similar conditions some decrease in the rate and maximum amount incorporated (20–50%) was obtained. The significance of this was doubtful owing to the variability of the effect and the precipitation of protein that usually occurred during the incubation. Tropomyosin did not significantly affect the rate of phosphorylation of troponin I although the maximum amount of phosphorus incorporated was increased somewhat (20–30%).

Biological activity and phosphorylation of the troponin complex

Rabbit skeletal muscle troponin that had been phosphorylated with phosphorylase kinase so that it contained 2.5–3mol of P/80000g was not significantly different from the original troponin in conferring EGTA sensitivity to the Mg^{2+}-stimulated ATPase of desensitized actomyosin over the range 0–150μg of troponin/mg of actomyosin (Fig. 7). At higher concentrations of troponin, however, it differed from the original troponin in no longer conferring EGTA sensitivity to the enzymic activity of desensitized actomyosin.

By treating rabbit skeletal muscle troponin with alkaline phosphatase of *E. coli* most of the covalently bound phosphate could be removed although it was not found possible to decrease the amount below 0.1–0.2mol/80000g. No difference from the original preparation could be detected in the biological activity of troponin dephosphorylated to this extent when assayed by the type of experiment shown in Fig. 7.

Phosphorylation of troponin in the absence of Ca^{2+}

Phosphorylase kinase. The phosphorylation of troponin by phosphorylase kinase required Ca^{2+} for maximum initial rates of incorporation. Nevertheless in the presence of EGTA phosphorylase kinase purified on Sepharose 4B still phosphorylated the troponin complex at a significant rate. This activity was not affected by the addition of 3′:5′-cyclic AMP. Under these conditions phosphorylase *b* was not phosphorylated by the enzyme (Fig. 8). As was the case when Ca^{2+} was present, phosphorylation of troponin by phosphorylase kinase in the presence of EGTA occurred mainly on troponin T, although a higher proportion of the total phosphate (up to 30%) was incorporated into troponin I. When the isolated components were incubated with phosphorylase kinase in the presence of EGTA troponin I was phosphorylated. The addition of troponin C inhibited this and inhibition was virtually complete at a molar ratio troponin C/troponin I of 1:1. Isolated troponin T, however, was phosphorylated extremely slowly under these conditions and the rate appeared to be unaffected by the addition of troponin C.

Bovine cardiac 3′:5′-cyclic AMP-dependent protein kinase. When incubated with a protein kinase isolated from ox heart (see under 'Materials') troponin from rabbit or chicken skeletal muscles was phosphorylated up to an additional 1.1mol of P/80000g. As with phosphorylase kinase the reaction was slow and usually several hours incubation under the conditions described in Fig. 9 were required for maximum incorporation. Phosphorylation by this enzyme was 3′:5′-cyclic AMP-dependent and usually more than 90% of the total phosphate incorporated was in the troponin I component, as judged from the distribution of radioactivity in the troponin components fractionated by polyacrylamide-gel electrophoresis in sodium dodecyl sulphate. It was concluded that there

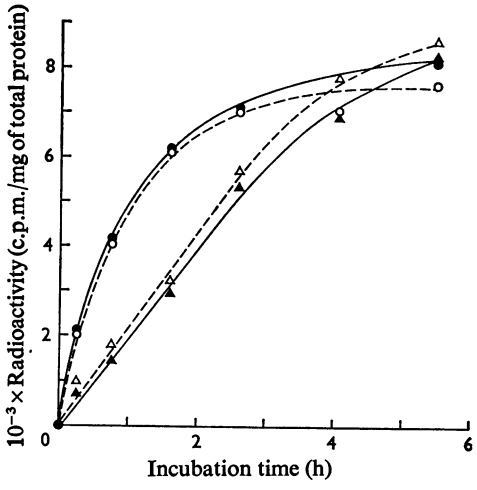


Fig. 8. *Action of phosphorylase kinase on troponin from rabbit skeletal muscle in the presence of EGTA*

Troponin preparations (0.6mg/ml) were incubated with phosphorylase kinase (0.09mg/ml) under standard conditions (see the Methods and Materials section) in 2mM-NaATP containing [γ-^{32}P]ATP (1.5μCi/ml) with 1mM-Ca^{2+} or 2.5mM-EGTA. The enzyme blank (less than 15% of the total) has been deducted from the values shown. ○, Troponin+Ca^{2+}+0.2mM-3′:5′-cyclic AMP; ●, troponin+Ca^{2+}; △, troponin+EGTA+0.2mM-3′:5′-cyclic AMP; ▲, troponin+EGTA.

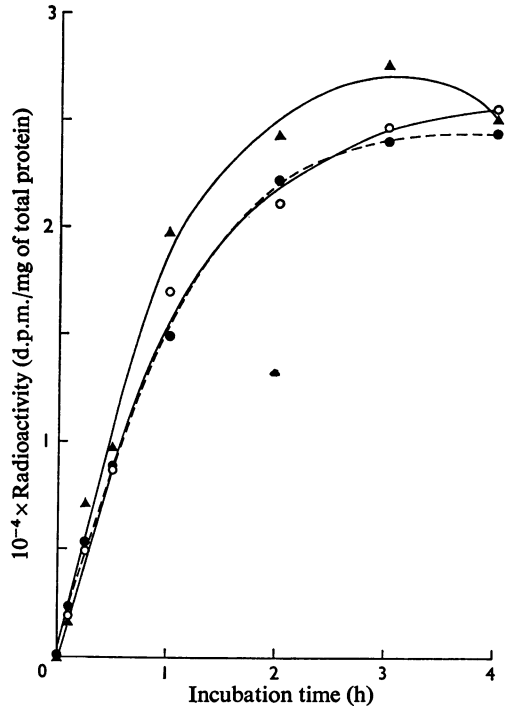


Fig. 9. *Phosphorylation of troponin from rabbit and chicken skeletal muscle by bovine cardiac protein kinase*

Troponin (0.4–0.6mg/ml) was incubated with bovine cardiac protein kinase [0.075mg/ml; Sigma (London) Chemical Co. Ltd.] at 30°C in 20mM-glycerophosphate (pH6.8), 10mM-magnesium acetate, 0.15mM-cyclic AMP, 2.5mM-EGTA, 25mM-NaF, 1mM-dithiothreitol and 2.5mM-NaATP (pH7.0) containing [γ-^{32}P]ATP (2.5μCi/ml). Samples were taken at the times shown and ^{32}P incorporation was determined as described in the Methods and Materials section. The enzyme blank (less than 5% of the total) has been deducted in each case. ○, Chicken breast muscle troponin; ●, chicken leg muscle troponin; ▲, rabbit mixed-skeletal-muscle troponin.

was no significant incorporation of phosphate into either troponin C or troponin T when this enzyme was incubated with troponin. Likewise when troponin T alone was incubated with bovine cardiac protein kinase no significant phosphorylation occurred.

Schwartz & Reddy (1973) found that cardiac troponin was phosphorylated by protein kinase. In our hands, with troponin from the rabbit heart this phosphorylation occurred mainly in troponin I and at a 5–10 times faster rate than that obtained with troponin from skeletal muscle.

Phosphorylated proteolytic fragments of troponin components

On incubation of troponin with purified phosphorylase kinase, particularly at pH6.8, it was noted on occasions that the amount of troponin T decreased and a protein component corresponding to an apparent molecular weight of 28000–30000, as judged by electrophoresis in sodium dodecyl sulphate, was produced. Less regularly a component of molecular weight about 22000 was also produced under these conditions. Both components were considered to be formed by degradation of troponin T owing to slight contamination of the preparations with a proteolytic enzyme. When phosphorylation was carried out in the presence of [γ-^{32}P]ATP the band of 28000–30000 molecular weight was invariably radioactive. Although it is clear that the phosphorylated component of 28000–30000 daltons was derived from troponin T (Dabrowska *et al.*, 1973) our investigations to date do not allow us to decide whether phosphorylation occurred before or after proteolytic degradation of the troponin T.

Discussion

The present study confirms and extends our preliminary report that the main substrate for the

action of phosphorylase kinase on the troponin complex *in vitro* is troponin T. The presence of approximately 1 mol of covalently bound phosphate/mol of troponin T and the further phosphorylation of this protein by phosphorylase kinase are useful criteria for identifying troponin T in the troponin complex. All the preparations of troponin and troponin T from skeletal muscle studied incorporated an additional 2 mol of phosphate/mol of protein on prolonged incubation with phosphorylase kinase, indicating that there are probably three sites of phosphorylation in the troponin T molecule.

The enzymic evidence suggests that there are also two sites for phosphorylation of troponin I by phosphorylase kinase and protein kinase. Study of peptides obtained by cyanogen bromide cleavage indicates that one of these sites is present in the *N*-terminal peptide and that threonine is the residue phosphorylated. The second site, which contains a serine residue and no threonine, is also phosphorylated by both enzymes (Moir *et al.*, 1974; Huang *et al.*, 1974). Thus there are potentially at least five sites/unit of troponin complex of rabbit white skeletal muscle that can be phosphorylated by protein kinase or phosphorylase kinase preparations.

The extent of phosphorylation of troponin I and troponin T is markedly modified by the presence of troponin C indicating that the latter component can interact with both proteins in such a way as to inhibit the phosphorylation by phosphorylase kinase. Although in the presence of troponin C only, the phosphorylation of both troponin I and troponin T is inhibited, in the whole troponin complex, phosphorylation of troponin I is preferentially inhibited if Ca^{2+} is present. Thus it may be concluded that although troponin C interacts with both troponin I and troponin T separately, when both are present and in the presence of Ca^{2+}, it preferentially interacts with troponin I.

The stoicheiometry of the interaction suggests that 1 mol of troponin C can block the phosphorylation sites of more than 1 mol of troponin I. It agrees well with the results of enzymic studies (Perry *et al.*, 1972) and immunochemical investigations (Hirabayashi & Perry, 1974) which indicate that at low ionic strength and in the absence of urea, 1 molecule of troponin C may interact with 2 molecules of troponin I.

The inhibitory action of troponin C on the phosphorylation of troponin I by phosphorylase kinase when excess of EGTA is present, implies that there is some interaction between troponin I and troponin C in the absence of Ca^{2+} (cf. Drabikowski *et al.*, 1973). The inhibition, and hence the interaction, was less than that obtained in the presence of Ca^{2+}, under which conditions a specific equimolar complex is formed (Head & Perry, 1974). The interaction obtained in the absence of Ca^{2+} may be due to the two molecules being oppositely charged at the pH values of the incubation media used. It did not prevent phosphorylation of troponin I in the troponin complex by bovine protein kinase.

Phosphorylated troponin I can still form a specific equimolar complex with troponin C in the presence of Ca^{2+}. This implies that phosphorylation itself does not prevent interaction and suggests that it occurs at sites on the troponin I molecule that are not directly involved in interaction with troponin C. A possible hypothesis is that this interaction leads to conformational changes in troponin I which render the phosphorylation sites unavailable to phosphorylase kinase.

The effect of troponin C on the phosphorylation of troponin I partially explains the discrepancy between our earlier results (Perry & Cole, 1973), confirmed by the present paper, and the report of Stull *et al.* (1972) that troponin I is the main site of phosphorylation by phosphorylase kinase. The latter investigators used as substrate troponin B (Hartshorne & Mueller, 1968), a mixture of troponin I and troponin T of variable composition (Wilkinson *et al.*, 1972). As troponin B contains little troponin C, troponin I will be phosphorylated, although in the light of our findings significant phosphorylation of troponin T would also be expected to occur. *In vivo* however, troponin I will normally be complexed with troponin C in which form the results *in vitro* suggest that it would be unavailable to the action of phosphorylase kinase.

Our limited studies do not as yet provide evidence of a biological role for the phosphorylation of the troponin complex. The rates of phosphorylation both by phosphorylase kinase from rabbit skeletal muscle and a 3′:5′-cyclic AMP-dependent protein kinase from bovine cardiac muscle are relatively slow compared with the rates obtained with these enzymes on their normal substrates. The question whether there exists in skeletal muscle another enzyme that can phosphorylate sites on troponin more effectively remains as yet unanswered. Certainly in our view the report of Gross & Mayer (1973), that troponin B can be phosphorylated by a phosphorylase kinase preparation, from I strain mice, which is inactive in phosphorylating phosphorylase *b*, indicates that this may be the case.

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