The Phosphorylation Sites of Troponin T from White Skeletal Muscle and the Effects of Interaction with Troponin C on their Phosphorylation by Phosphorylase Kinase

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1. The phosphorylation of troponin T from rabbit white skeletal muscle is catalysed by phosphorylase kinase, but not at a significant rate by bovine 3':5'-cyclic AMP-dependent protein kinase. 2. The amino acid sequences adjacent to the three major phosphorylation sites of troponin T were determined. 3. The serine in the *N*-terminal peptide (Asx,SerP,Glx)Glu-Val-Glu, is that phosphorylated (SerP, phosphoserine) when the troponin complex is isolated. 4. The other two sites of phosphorylation are located in the sequence Ala-Leu-(Ser,SerP)-Met-Gly-Ala-Asn-Tyr(Ser,SerP)Tyr. 5. When troponin T is phosphorylated in the presence of troponin C, the extent of phosphorylation at each site is considerably decreased. 6. CNBr fragments of troponin T are also phosphorylated by phosphorylase kinase, but the rate of phosphorylation at each site in the CNBr fragments is considerably slower than in the native protein. 7. From these studies it is suggested that troponin C interacts with troponin T in the region containing the two closely situated phosphorylation sites.

The regulation of contractile activity in the myofibril requires both tropomyosin and troponin, a complex of three distinct proteins, troponin C, troponin I and troponin T. The roles of troponin C and troponin I, as the proteins involved in binding Ca^{2+} and inhibiting the actomyosin ATPase* respectively, are more clearly defined than that of troponin T. Troponin T, since it interacts with tropomyosin and troponin C, may have the function of localizing the troponin complex at defined sites along the tropomyosin filaments lying in the grooves of the actin helix of the I filament. At the same time it could transmit to the tropomyosin conformational changes initiated in the troponin complex by the binding of Ca^{2+} . This function may not be essential for Ca^{2+} sensitization of the Mg^{2+} -stimulated ATPase, since, under some circumstances, actomyosin ATPase systems that are sensitive to Ca^{2+} can be reconstituted in the absence of troponin T (Perry *et al.*, 1972; Amphlett, *et al.*, 1977).

Troponin T is one of the proteins of the troponin complex that can be phosphorylated by enzymes present in skeletal muscle. The process can occur *in vivo* on one site at least, for most of the covalently bound phosphate present in troponin isolated from rabbit white skeletal muscle by the procedure of Ebashi *et al.* (1971) is associated with troponin T (Perry & Cole, 1973). On prolonged incubation with phosphorylase kinase, a total of 3 mol of phosphate/

mol of troponin T can be incorporated, suggesting that three sites of phosphorylation are present (Perry & Cole, 1974).

The physiological function of the phosphorylation of troponin T is, as yet, a matter for speculation. Nevertheless, investigation of the phosphorylation sites of troponin T in relation to its primary structure, and the effect of troponin C on their ability to be phosphorylated, can provide useful information about the sites of interaction of troponin C and troponin T. The present paper describes such a study.

Three sites specific for phosphorylase kinase have been identified and an examination of their phosphorylation in the presence and absence of troponin C suggests that interaction with troponin C involves a region of the molecule of troponin T close to that interacting with tropomyosin. Some aspects of the work have been briefly reported (Perry *et al.*, 1975, 1976).

Materials and Methods

Preparation of troponin and components

Troponin was prepared from rabbit white skeletal muscle by the method of Ebashi *et al.* (1971) and fractionated into troponin C, troponin I and troponin T by chromatography on DEAE-cellulose as described by Perry & Cole (1974) or by chromatography on CM-cellulose after fractionation into troponin A and troponin B (Wilkinson, 1974).

* Abbreviation: ATPase, adenosine triphosphatase.

Preparation of phosphorylase kinase

Phosphorylase *b* kinase was prepared from rabbit white skeletal muscle by the method of Cohen (1973). The peak obtained by gel filtration that contained phosphorylase kinase activity was dialysed against 20% (w/v) sucrose and stored at -20°C (Cole & Perry, 1975).

Incubations with phosphorylase kinase

For experiments in which the incorporation of ^{32}P in the whole protein was measured, conditions were, unless specifically indicated in the text, as described by Cole & Perry (1975). When the ^{32}P -labelled troponin T was required for subsequent peptide isolation, the enzymic reaction was stopped by the addition of either an equal volume of 15% (w/v) trichloroacetic acid, or solid guanidine hydrochloride to give a final concentration of approx. 6M. After the addition of guanidine hydrochloride, the pH was lowered to 2.0 by the addition of 1 M-HCl and the phosphorylated troponin T isolated by gel filtration on a column (2.5 cm $\times$ 110 cm) of Sephadex G-50 or G-75 equilibrated against 10 mM-HCl. This enabled the isolation of troponin T free from troponin C when this protein was added for interaction studies.

Determination of phosphate and protein

Total phosphate and ^{32}P content were determined as described by Perry & Cole (1974). Protein was measured by a method involving Nesslerization after digestion (Strauch, 1965), assuming that the protein contained 16% N.

Digestion of ^{32}P -labelled troponin T with CNBr

Troponin T was dissolved in 0.1 M-HCl (10–20 mg/ml) and solid CNBr was added to give a 100-fold molar excess of CNBr over the total number of methionine residues present. The mixture was left at 23°C for 20 h and then freeze-dried without the addition of water.

Separation of the CNBr peptides

Freeze-dried CNBr digests of troponin T were dissolved in 0.01 M-HCl (20–40 mg/ml) and applied to a column (2.5 cm $\times$ 110 cm) of Sephadex G-75 equilibrated with 0.01 M-HCl. Peptides were eluted in 5 ml fractions and were located in the eluate by measuring the A_{215} . Peptides containing ^{32}P were located by measuring the radioactivity in every second 5 ml fraction by the Čerenkov procedure. Individual CNBr peptides were isolated as necessary by the methods of Jackson *et al.* (1975), whose nomenclature for the peptides has been adopted throughout (Fig. 1).

Fig. 1. Arrangement of peptides obtained by CNBr digestion of troponin T of rabbit skeletal muscle

From Jackson *et al.* (1975). P represents the positions of serine residues that can be phosphorylated by phosphorylase kinase.

Enzymic digestion of troponin T and of peptides derived from troponin T

^{32}P -labelled troponin T and the CNBr peptides derived from it were dissolved in 1% (w/v) NH_4HCO_3 , pH 7.9 (0.1–0.25 μmol in 1.0 ml), and 2% of their weight of trypsin was added. Digestion was allowed to proceed for 4 h at 37°C or for 16 h at 30°C and the digests were freeze-dried.

Tryptic peptides of troponin T (0.1–0.2 μmol) were further digested with thermolysin in 1% (w/v) NH_4HCO_3 , or with pepsin in 0.01 M-HCl, at an enzyme:peptide molar ratio of 1:50 in both cases in a total volume of 1.0 ml. Digestion was performed for 2 h at 37°C , and the samples were freeze-dried.

High-voltage paper electrophoresis

High-voltage paper electrophoresis of peptides obtained from enzymic digestion of either ^{32}P -labelled troponin T or the CNBr peptides of ^{32}P -labelled troponin T was performed on Whatman 3MM chromatography paper at pH 6.5, 3.5 or 2.0, by using the electrophoresis buffers described by Wilkinson (1974). The potential gradient applied was 40–50 V/cm.

Peptides were located by staining side strips with a cadmium/ninhydrin reagent (Heilmann *et al.*, 1957) and eluted by descending chromatography in 50 mM- NH_3 (for acidic or neutral peptides) or 5% (v/v) acetic acid (for basic peptides).

The mobility of peptides at pH 6.5 was expressed relative to the mobility of aspartic acid ($m_{6.5} = +1$), the position of serine representing zero mobility at pH 6.5; mobilities at pH 2.0 were calculated relative to the mobility of serine ($m_{2.0} = +1$), 5-dimethylaminonaphthalene-1-sulphonic acid representing zero mobility at pH 2.0.

Location of radioactive peptides

The positions of ^{32}P -labelled peptides on electrophoresis sheets were found by radioautography, by

using Kodak Blue Brand medical X-ray film (BB 54) with exposure times of 12–90h.

Amino acid analysis

Peptides were hydrolysed *in vacuo* in 6M-HCl at 110°C for 20h, and the HCl was then removed by rotary evaporation. Hydrolysates containing homoserine lactone were subsequently heated at 100°C for 1h in pyridine/acetic acid/water (25:1:225, by vol.), pH6.5 (Ambler, 1965), to convert the lactone into homoserine.

Amino acid analysis was performed on a Beckman 120B or a Locarte amino acid analyser.

Sequence determination

Amino acid sequences were determined by the dansyl-Edman procedure of Hartley (1970), by using 5cm×5cm polyamide layers (BDH Chemicals, Poole, Dorset BH12 4NN, U.K.) to identify the dansyl-amino acids.

Materials

Trypsin (treated with 1-chloro-4-phenyl-3-L-tosylamidobutan-2-one) and pepsin were from Cambrian Chemicals, Croydon CR0 4XB, Surrey, U.K. Bovine cardiac 3':5'-cyclic AMP-dependent protein kinase and thermolysin (thermophilic - bacterial proteinase, type X) were from Sigma Chemical Co., Kingston-upon-Thames, Surrey KT2 7BH, U.K.

[γ -³²P]ATP was from The Radiochemical Centre, Amersham, Bucks., U.K., as the freeze-dried NH₄⁺ salt.

Sephadex G-50 and G-75 were from Pharmacia Fine Chemicals AB, Uppsala, Sweden.

Results

Phosphate content of troponin T

When isolated from the troponin complex, troponin T of rabbit white skeletal muscle is usually phosphorylated. In an earlier report (Perry & Cole, 1973) the average phosphate content was given as 0.74 mol of P/mol based on an apparent mol.wt. of 37000, derived from electrophoresis in sodium dodecyl sulphate. It now appears that the molecular weight of troponin T is much lower (Jackson *et al.*, 1975) and from the complete amino acid sequence the value has been estimated to be 30500 (L. B. Smillie, personal communication). On the basis of this molecular weight, isolated troponin T contains on average 0.61 mol of P/mol (Table 1). Phosphate analysis of the CNBr peptides of troponin T isolated as described by Jackson *et al.* (1975) indicated that virtually all the endogenous phosphate is present in peptide CNB1, the N-terminal peptide (Table 1).

Phosphorylation of troponin T *in vitro*

The rate of phosphorylation of troponin T *in vitro*

Table 1. *Phosphate content of rabbit white-skeletal-muscle troponin T and the CNBr peptides derived from it*

For peptide nomenclature see Fig. 1. Troponin T and the CNBr peptides derived from it were prepared as described in the Materials and Methods section. With the exception of peptide CNB5, from which salt had been removed by gel-filtration chromatography on Sephadex G-15 (Jackson *et al.*, 1975), they were precipitated and washed repeatedly with 7.5% (w/v) trichloroacetic acid. The phosphate content was then determined as described by Perry & Cole (1974). Assumed molecular weights of peptides are from Jackson *et al.* (1975).

Protein	Phosphate content (mol/mol)	Assumed mol.wt.
Troponin T	0.61	30500
CNB1'	0.56	19000
CNB1	0.77	8400
CNB2	0.05	9500
CNB5	0.01	3000
CNB6	0.1	8000

Fig. 2. Comparison of the rates of phosphorylation of troponin I and troponin T catalysed by 3':5'-cyclic AMP-dependent protein kinase

Approximately equimolar solutions of rabbit white-skeletal-muscle troponin I (0.16mg/ml) and troponin T (0.25mg/ml), previously dialysed against 50mM-KCl, were incubated at 30°C with bovine cardiac 3':5'-cyclic AMP-dependent protein kinase (0.04mg/ml) and [γ -³²P]ATP (1.25mM; 1 μ Ci/ml) under standard conditions (see the Materials and Methods section), with the addition of 20mM-KCl and either 0.5mM-CaCl₂ or 2mM-EGTA. Samples (1ml) were precipitated with 0.5ml of 15% (w/v) trichloroacetic acid, and 0.1ml of 1% (w/v) bovine serum albumin was added before the precipitates were washed and assayed for radioactivity by the usual procedure. The enzyme blank (approx. 300d.p.m.) has been deducted to give the values shown. ○, Troponin I+EGTA; ●, troponin I+CaCl₂; △, troponin T+EGTA; ▲, troponin T+CaCl₂.

by bovine cardiac 3':5'-cyclic AMP-dependent protein kinase was low compared with that obtained with troponin I, also from rabbit white skeletal muscle, as substrate (Fig. 2). It was barely significant compared with the initial rate at which phosphorylase kinase catalysed the phosphorylation of troponin T. This initial rate was at least equal to that obtained under similar conditions with troponin I as substrate (Fig. 3). The total amount of phosphate incorporated into troponin T, however, was greater. The phosphorylation of troponin T occurred, even though it contained 0.6 mol of endogenous phosphate/mol, and on prolonged incubation the total phosphate content rose to nearly 3 mol of P/mol (Perry & Cole, 1974).

Phosphorylation catalysed by phosphorylase kinase preparations was strongly inhibited by EGTA, although a small amount of phosphorylation did occur in the presence of 2 mM-EGTA.

Tryptic digestion of ^{32}P -labelled troponin T

Troponin T that had previously been phosphorylated by prolonged incubation with phosphorylase kinase and $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ was digested with trypsin (see the Materials and Methods section) and the digest fractionated by gel filtration on a column (2.5 cm $\times$ 110 cm) of Sephadex G-50 equilibrated with 0.01 M-HCl (Fig. 4). Radioactivity was associated with two portions of the eluate, fractions A and B (Fig. 4).

On examination by paper electrophoresis at pH 6.5 and 2.0 fraction A was found to consist of a single peptide, peptide TA, which contained the radioactivity. Its amino acid composition and ^{32}P content are given in Table 2. When peptide TA was digested with pepsin and the digest fractionated by electrophoresis at pH 2.0, a single peptide containing radioactivity, peptide TA-PEP1, was obtained. The amino acid composition of this peptide is also given in Table 2.

Peptide TA-PEP1 had a negative charge on electrophoresis at pH 2.0 and was not stained with ninhydrin. Both observations indicated that the *N*-terminal residue of peptide TA-PEP1 was blocked. Wilkinson (1974) has shown that the *N*-terminal sequence of rabbit skeletal troponin T is X(Asx,Ser,Glx)Glu-Val-Glu, where X represents an *N*-terminal blocking group. The similarities between this sequence and the amino acid analysis of peptide TA-PEP1 indicated that the phosphorylated peptide was derived from the *N*-terminus of troponin T.

Although peptides TA and TA-PEP1 were fully phosphorylated, from the determination of their ^{32}P content they had incorporated only 0.11 and 0.16 mol of phosphate/mol respectively during incubation with phosphorylase kinase and $[\gamma\text{-}^{32}\text{P}]\text{ATP}$. Consequently both must have contained endogenous unlabelled phosphate before phosphorylation with phosphorylase kinase.

Fig. 3. Comparison of the rates of phosphorylation of troponin I and troponin T catalysed by phosphorylase kinase

Approximately equimolar amounts of rabbit white-skeletal-muscle troponin I (0.1 mg/ml) and troponin T (0.15 mg/ml) were incubated with phosphorylase kinase (0.06 mg/ml) and $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (1.25 mM; 1.3 $\mu\text{Ci/ml}$) under standard conditions (see the Materials and Methods section) with the addition of 20 mM-KCl and either 0.5 mM- CaCl_2 or 2 mM-EGTA. The radioactivity incorporated in the phosphorylase kinase (600–1200 d.p.m./ml of incubation mixture) has been deducted to give the values shown. ▲, Troponin T + CaCl_2 ; △, troponin T + EGTA; ●, troponin I + CaCl_2 ; ○, troponin I + EGTA.

Fraction B was examined by paper electrophoresis at pH 6.5, 3.5 and 2.0, and two different ^{32}P -containing peptides could be located by radioautography. Only one of these, peptide TB1, could be purified by further electrophoresis at pH 6.5 and 2.0 to a satisfactory state of purity for further analysis. Its amino acid composition and ^{32}P content are given in Table 2. Digestion of peptide TB1 with thermolysin gave two smaller peptides, TB-THE1 and TB-THE2 (Table 2), of which only peptide TB-THE1 contained radioactivity. The amino acid sequence of peptide TB1

Fig. 4. *Chromatography of a tryptic digest of ^{32}P -labelled troponin T on Sephadex G-50*

A tryptic digest of ^{32}P -labelled troponin T (approx. 100mg) dissolved in about 6ml of 0.01M-HCl was applied to a column (2.5cm $\times$ 110cm) of Sephadex G-50 that was equilibrated in 0.01M-HCl, and eluted with 0.01M-HCl; 5ml fractions were collected. The A_{230} of each sample was measured and radioactivity measured by the Čerenkov procedure. Samples were pooled into fractions A and B as indicated by the horizontal bars. —, A_{230} ; ----, ^{32}P radioactivity (c.p.m.).

Table 2. *Amino acid compositions of phosphorylated peptides TA and TB1 isolated from enzymic digests of ^{32}P -labelled troponin T*

The values for each amino acid are expressed as mol/mol of peptide. Amounts of any amino acid which were present at less than 0.20mol/mol have not been included in this Table. The ^{32}P content of each peptide is expressed as mol of ^{32}P /mol of peptide, and was determined from the amount of ^{32}P in each peptide after elution. Values in parentheses are nearest whole numbers of residues. N.D. indicates that the mobility of the peptide was not determined at that pH.

Peptide ...	TA	TB1	TA-PEP1	TB-THE1	TB-THE2
Asp	0.90 (1)	1.09 (1)	1.00 (1)	— —	1.10 (1)
Ser	1.97 (2)	1.94 (2)	0.84 (1)	1.78 (2)	— —
Glu	18.18 (18)	0.25 —	2.12 (2)	— —	— —
Pro	7.32 (7)	— —	— —	— —	— —
Gly	— —	1.02 (1)	— —	1.23 (1)	— —
Ala	4.16 (4)	1.69 (2)	— —	0.84 (1)	0.85 (1)
Val	5.07 (5)	— —	— —	— —	— —
Met	— —	0.65 (1)	— —	1.11 (1)	— —
Leu	— —	1.27 (1)	— —	1.07 (1)	— —
Tyr	— —	0.97 (1)	— —	— —	1.18 (1)
His	2.65 (3)	— —	— —	— —	— —
Lys	2.22 (2)	— —	— —	— —	— —
Arg	0.98 (1)	— —	— —	— —	— —
^{32}P content (mol/mol)	0.13	0.96	0.16	0.93	0
* $m_{6.5}$	0.72	0.37	N.D.	0.49	0
* $m_{2.0}$	0.89	0.07	-0.40	N.D.	0.85

* Electrophoretic mobilities at the pH values indicated.

was deduced from the sequences of these peptides to be Ala-Leu(Ser,SerP)Met-Gly-Ala-Asn-Tyr (where SerP represents phosphoserine). The aspartic acid residue at position 8 in this sequence was considered to be in the amide form, as the mobility of the tripeptide TB-THE2 at pH 6.5 was zero.

The ^{32}P content of peptide TB1 indicated that about 0.90–0.96 mol/mol had been transferred from $[\gamma\text{-}^{32}\text{P}]\text{-ATP}$ on incubation of troponin T with phosphorylase kinase. This implies that, unlike the *N*-terminal phosphorylation site, troponin T was not phosphorylated to any significant extent at this position in the original sample of troponin T. The sequence of peptide TB1 is identical with that reported by

Jackson *et al.* (1975) for the peptide-bridging peptides CNB2 and CNB5 obtained by CNBr digestion of troponin T from rabbit white muscle (Fig. 1). From the distribution of ^{32}P in the sub-peptides of TB1 it was concluded that the phosphorylation site in peptide TB1 was one of the two serine residues adjacent to the methionine residue.

CNBr digestion of ^{32}P -labelled troponin T

Although analysis of tryptic digests of ^{32}P -labelled troponin T provided evidence of three different sites of phosphorylation, only two peptides could be purified and definitively analysed from these digests. The remaining site was identified by investigation of the

Fig. 5. Gel filtration of a CNBr digest of ^{32}P -labelled troponin T on Sephadex G-75

A CNBr digest of ^{32}P -labelled troponin T (approx. 25 mg) dissolved in 2 ml of 6M-guanidinium hydrochloride/0.4M-Tris/1 mM-dithiothreitol, pH 8.0, was applied to a column (2.5 cm $\times$ 110 cm) of Sephadex G-75 equilibrated in 0.01 M-HCl, and eluted with 0.01 M-HCl; 5 ml fractions were collected, and their A_{215} values measured. Radioactivity was measured in the fractions by the Čerenkov procedure. Samples were pooled into fractions 1, 2 and 3 as indicated by the horizontal bars. —, A_{215} ; ----, ^{32}P radioactivity (c.p.m.).

peptides obtained when ^{32}P -labelled troponin T was digested with CNBr. Gel filtration of the whole CNBr digest of ^{32}P -labelled troponin T on a column (2.5 cm $\times$ 110 cm) of Sephadex G-75, equilibrated against 0.01 M-HCl, yielded three fractions containing ^{32}P , fractions 1, 2 and 3 (Fig. 5).

Fractions 1 and 2 were dissolved in 0.2 M-ammonium acetate, pH 3.5, and re-chromatographed on CM-cellulose equilibrated against the same buffer, and fraction 3 was chromatographed on CM-cellulose in a sodium phosphate buffer system at pH 7.5 [fractionation system 4 described by Jackson *et al.* (1975)].

Fractions 1 and 3 each contained a single phosphorylated peptide, identical with peptides CNB1' and CNB5 (Jackson *et al.*, 1975) respectively, and fraction 2 contained peptides CNB1 and CNB2, both of which were radioactive. The amino acid composition of each of these peptides is presented in Table 3. Jackson *et al.* (1975) have shown that peptide CNB1 is the *N*-terminal CNBr peptide of troponin T, consisting of approx. 70 residues, and peptides CNB2 and CNB5 are respectively the second and third peptides from the *N*-terminus of troponin T. Peptide CNB1' is a partial digestion product, resulting from incomplete cleavage at the methionine residue closest to the *N*-terminus of troponin T, and contains both peptides CNB1 and CNB2 (Jackson *et al.*, 1975).

Digestion of peptide CNB1 with pepsin produced only one radioactive peptide, which had a mobility of -0.35 on electrophoresis at pH 2.0. This radioactive peptide had a blocked *N*-terminal residue and its

amino acid content was identical with that of peptide TA-PEP1 listed in Table 2. This confirmed that the site of phosphorylation in peptide CNB1 is that located at the *N*-terminus of the peptide and which was also isolated from tryptic digests of ^{32}P -labelled troponin T (see above).

Tryptic digestion of peptide CNB2 produced only one peptide containing radioactivity that also contained homoserine, suggesting that it was derived from the *C*-terminus of peptide CNB2. This confirms the conclusion made from the study of the tryptic peptide TB1, that the phosphorylation site in the CNBr fragment CNB2 is close to the *C*-terminus.

Digestion of peptide CNB5 with trypsin produced a single peptide containing radioactivity, which was isolated by paper electrophoresis at pH 6.5 and then pH 2.0. The amino acid composition of this phosphorylated peptide was Asp (1.34 residues), Ser (1.88), Gly (1.10), Ala (1.15), Tyr (1.54), and glycine was found to be the *N*-terminal amino acid by the dansyl procedure. CNBr peptide CNB5 contains only two serine residues (Table 3), and its *N*-terminal sequence has been shown to be Gly-Ala-Asn-Tyr-Ser-Ser-Tyr (Jackson *et al.*, 1975; Collins, 1975). From the *N*-terminal analysis and amino acid composition it can be concluded that the phosphorylated peptide obtained by tryptic digestion of peptide CNB5 must be derived from the *N*-terminus. Therefore we conclude that one of the two serine residues near the *N*-terminus of peptide CNB5 can be phosphorylated by phosphorylase kinase.

We have shown above that the sequence at the *C*-

Table 3. Amino acid composition of the major phosphorylated peptides obtained from CNBr digestion of ^{32}P -labelled troponin T

The values are expressed as mol/mol of peptide, except for homoserine, which was incompletely resolved from glutamic acid. Values in parentheses are the nearest whole numbers of residues. Amounts of any amino acids present at less than 0.20 mol/mol have not been included. See Fig. 1 for peptide nomenclature.

Peptide ...	CNB1'	CNB1	CNB2	CNB5
Asp	11.0 (11)	6.38 (6)	4.60 (5)	1.90 (2)
Thr	1.01 (1)	1.15 (1)	—	1.08 (1)
Ser	4.98 (5)	1.93 (2)	2.68 (3)	1.71 (2)
Glu	43.29 (43)	21.18 (21)	23.88 (24)	3.32 (3)
Pro	10.16 (10)	9.20 (9)	—	—
Gly	2.50 (3)	1.27 (1)	—	2.15 (2)
Ala	15.01 (15)	4.94 (5)	11.06 (11)	3.98 (4)
Val	6.69 (7)	6.28 (6)	1.24 (1)	—
Ile	5.84 (6)	2.14 (2)	2.99 (3)	—
Leu	10.75 (11)	2.23 (2)	7.29 (7)	1.19 (1)
Tyr	—	—	—	1.60 (2)
Phe	2.11 (2)	1.04 (1)	1.09 (1)	—
His	4.09 (4)	3.42 (3)	1.12 (1)	—
Lys	19.40 (19)	6.91 (7)	11.27 (11)	4.01 (4)
Arg	16.62 (17)	2.01 (2)	12.90 (13)	1.81 (2)
Hse*	+	+	+	+

* Abbreviation: Hse, homoserine; present, but actual amounts not determined.

terminus of the phosphorylated peptide TB1 is Met-Gly-Ala-Asn-Tyr. Thus two of the three phosphorylation sites in troponin T are located close together in the sequence Ala-Leu(Ser,SerP)Met-Gly-Ala-Asn-Tyr(SerP,Ser)Tyr, the methionine residue in this sequence being located at about position 150 from the *N*-terminus of troponin T (Jackson *et al.*, 1975).

Phosphorylation of CNBr peptides of troponin T

The CNBr digest of troponin T was a much less satisfactory substrate for phosphorylase kinase than was the whole protein. When phosphorylated digests were examined by electrophoresis or by gel filtration, ^{32}P could be detected in the bands corresponding to peptides CNB1', CNB1, CNB2 and CNB5. When tested separately as substrates, the isolated cyanogen bromide peptides were phosphorylated at about 2–7% of the rate of troponin T (Fig. 6).

Fig. 6. Phosphorylation of troponin T and its CNBr peptides catalysed by phosphorylase kinase

Rabbit mixed-skeletal-muscle troponin T (○; 0.2 mg/ml) and the CNBr peptides CNB1 (●), CNB2 (▲) and CNB1' (△) (0.7, 0.9 and 0.8 mg/ml respectively) were incubated with phosphorylase kinase (0.30 mg/ml) and $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (1.25 mM; 2.5 $\mu\text{Ci/ml}$) under the standard conditions for this enzyme (see the Materials and Methods section). Samples were taken at the times indicated into an equal volume of 15% (w/v) trichloroacetic acid and the precipitates were washed with 7.5% (w/v) trichloroacetic acid. The blank corresponding to phosphate incorporation into phosphorylase kinase (less than 15% of that incorporated into troponin T) has been deducted to give the values shown.

Effect of interaction with troponin C on the phosphorylation of troponin T

Although troponin T and troponin I are both substrates for phosphorylase kinase, troponin T is the only component phosphorylated to a significant extent when the troponin complex is incubated with the enzyme (Perry & Cole, 1974). This effect is observed because the interaction of troponin I with troponin C renders the phosphorylation sites on troponin I unavailable to the enzyme (Perry & Cole, 1974). As troponin T also interacts with troponin C (Ebashi *et al.*, 1972; van Eerd & Kawasaki, 1973; Perry & Cole, 1974; Jackson *et al.*, 1975), the effect of troponin C on the phosphorylation of troponin T can be used to obtain information about the regions

Fig. 7. Effect of troponin C on the phosphorylation of troponin T catalysed by phosphorylase kinase

Rabbit skeletal-muscle troponin T (0.4 mg/ml) was incubated with various amounts of rabbit skeletal-muscle troponin C at the molar ratios indicated (assuming mol. wts. of 30 500 and 18 000 respectively), phosphorylase kinase (0.05 mg/ml) and $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (2.5 mM; 2.5 $\mu\text{Ci/ml}$) in the presence of either 0.5 mM- CaCl_2 (▲) or 2.5 mM-EGTA (△) under standard conditions (see the Materials and Methods section) in a final volume of 1 ml; 0.5 ml of 15% (w/v) trichloroacetic acid was added after 50 min if CaCl_2 was present and after 100 min if EGTA was present, and the concentration of troponin C was made equal throughout before the precipitates were washed and assayed for radioactivity by the usual procedure. The enzyme blanks (1300 d.p.m./mg with CaCl_2 and 850 d.p.m./mg with EGTA) have been deducted to give the values shown.

Fig. 8. Gel filtration of CNBr digests of troponin T phosphorylated in the presence or absence of troponin C

Samples of troponin T (12mg) were phosphorylated in the absence or in the presence of troponin C (9mg) as described in the Materials and Methods section. The ³²P-labelled troponin T samples were purified by gel filtration and digested with CNBr. Identical amounts of the digests of the two phosphorylated samples were dissolved in approx. 2ml of 0.01M-HCl and fractionated on Sephadex G-75 (2.5cm×110cm) in 0.01M-HCl. —, Troponin T phosphorylated in absence of troponin C; ----, troponin T phosphorylated in presence of troponin C.

of troponin T involved in the interaction with troponin C.

With increasing molar ratios of troponin C to troponin T, phosphorylation of the latter protein was progressively inhibited. The maximum inhibition was about 90% and, particularly with freshly prepared proteins, was reached when the molar ratio was 1:1 (Fig. 7). With some preparations slightly higher molar ratios of troponin C were required for maximum inhibition. The reason for this variation is not clear, but it may be a feature of the troponin T

and possibly also the troponin C preparations. In our experience, troponin T preparations can be somewhat variable in their solubility, and this was often modified by the type of preparative and storage procedures to which the protein had been subjected. After several months' storage, troponin C preparations did not inhibit phosphorylation of troponin T quite so effectively as did freshly prepared troponin C.

A relatively low rate of phosphorylation of troponin T by phosphorylase kinase, of about 6% of the normal rate, occurred in the presence of EGTA, under

Table 4. *Effect of troponin C on the phosphorylation of troponin T*

Troponin T was phosphorylated as described in the Materials and Methods section by using phosphorylase kinase and [γ - 32 P]ATP in the presence and absence of the amounts of troponin C indicated. Each sample of 32 P-labelled troponin T was isolated by gel filtration on Sephadex G-75, and digested with CNBr in 0.1 M-HCl. The CNBr digests were fractionated by gel filtration on Sephadex G-75 (as illustrated in Fig. 8) and each of the CNBr peptides was purified by the methods of Jackson *et al.* (1975). Inhibition (%) = $100 \times (^{32}\text{P incorporated in absence of troponin C} - ^{32}\text{P incorporated in presence of troponin C}) / ^{32}\text{P incorporated in absence of troponin C}$.

Molar ratio of troponin T:troponin C	Inhibition (%)		
	Site I (CNB1)	Site II (CNB2)	Site III (CNB5)
1:0.8	87	58	79
1:1	81	72	72
1:1.3	90	90	98

which conditions phosphorylase *b* was also phosphorylated at about 6% of its rate in the presence of Ca^{2+} . This lower rate of phosphorylation of troponin T was not significantly changed by increasing molar ratios of troponin C.

Effect of interaction with troponin C on the phosphorylation at each site of troponin T

Troponin T was phosphorylated with [γ - 32 P]ATP and phosphorylase kinase in the presence and absence of various amounts of troponin C under otherwise standard conditions. With molar ratios of troponin T to troponin C of 1:0.8, 1:1.0 and 1:1.3 the extent of inhibition of phosphorylation of troponin T was 70.9, 81.0 and 95.1% respectively of the values obtained in the absence of troponin C.

Each of these six samples of 32 P-labelled troponin T was purified by gel filtration and digested with CNBr. The digests were fractionated on a column (2.5 cm $\times$ 110 cm) of Sephadex G-75 equilibrated in 0.01 M-HCl. The elution profiles obtained for the CNBr digests of troponin T phosphorylated in the presence and absence of a 1:1.3 molar ratio of troponin C are shown in Fig. 8. They demonstrate that the incorporation of phosphate into each of the fractions of the CNBr digest of troponin T was markedly decreased in the presence of troponin C. The three major phosphorylated peptides were purified as described above from each digest and the amount of 32 P incorporated into each was determined. The degree of inhibition of phosphorylation at each site in troponin T by each concentration of troponin C is presented in Table 4.

Discussion

The identification of three phosphorylation sites on troponin T, sites I, II and III (Table 5), correlates well with the observation (Perry & Cole, 1973) that on prolonged incubation with phosphorylase kinase a maximum of about 3 mol of phosphate/mol are incorporated into troponin T from white skeletal muscle of the rabbit. The specific radioactivities of the 32 P-labelled peptides isolated from digests of troponin T phosphorylated *in vitro* indicate that only the N-terminal site I was significantly phosphorylated before the phosphorylation *in vitro*. The data indicate that this site is 60–70% phosphorylated in troponin T prepared by the procedure of Ebashi *et al.* (1971) from rabbit white skeletal muscle and accounts for the major part of the covalently bound phosphate present in troponin. Presumably this site is phosphorylated *in vivo* and is not readily dephosphorylated by endogenous phosphatase during isolation. There is little evidence as to whether sites II and III are phosphorylated *in vivo*, but lose their phosphate during the isolation procedure. The amounts of 32 P incorporated into site III during incubations of peptide CNB5 with phosphorylase kinase *in vitro* suggested that up to 5% of the serine residues at this site may be phosphorylated in some preparations of troponin T.

Partial phosphorylation of site I would explain the observation of Wilkinson (1974) that, on electrophoresis at pH 6.5, the N-terminal peptide obtained by Pronase digestion of troponin T migrated as two distinct bands that had the same amino acid compositions. The molar ratio of the faster (phosphorylated) to slower (unphosphorylated) peptides was 4:1, which is consistent with the extent of phosphorylation observed in the present study.

Comparison of all the sequences about the serine and threonine residues in troponin I and troponin T, the phosphorylation of which has been reported to be catalysed by phosphorylase kinase, suggests that this kinase has a considerably broader specificity than was previously supposed (Table 5). The only sequence that shows any degree of similarity to the phosphorylated sequence in phosphorylase *a* is that adjacent to threonine-11 in troponin I (Moir *et al.*, 1974; Huang *et al.*, 1974). In assessing these results it should be emphasized that the initial rates of phosphorylation of troponin T and troponin I by phosphorylase kinase are usually less than 5% of the rate of phosphorylation of phosphorylase *b* under similar conditions (Perry & Cole, 1973; Cole & Perry, 1975). The possibility cannot be ignored that the purified preparation of phosphorylase kinase used in the present study contains traces of other kinases specific for certain sites on troponin I or troponin T. The existence of such an enzyme or enzymes is suggested by the fact that troponin T isolated from I-

Table 5. *Amino acid sequences of peptides from troponin T, troponin I and phosphorylase that are phosphorylated by phosphorylase kinase*

The X in one sequence from troponin T indicates an *N*-terminal blocking group. References: ¹ Moir *et al.* (1974), Huang *et al.* (1974); ² Nolan *et al.* (1964). ThrP, phosphothreonine.

Source	Sequence
Troponin T (site I)	X(Asx, Glx, SerP)Glu-Val-Glu
Troponin T (site II)	Ala-Leu(SerP, Ser)Met-Gly-Ala
Troponin T (site III)	Asn-Tyr(SerP, Ser)Tyr
Troponin I	Arg-Met-SerP-Ala-Asp-Ala-Met ¹
Troponin I	Ala-Ile-ThrP-Ala-Arg-Arg-Gln ¹
Phosphorylase	Gln-Ile-SerP-Val-Arg ²

strain mice is partially phosphorylated (P. Cohen, H. A. Cole & S. V. Perry, unpublished work) and that troponin B can be phosphorylated by a phosphorylase kinase preparation from I-strain mice that will not catalyse the phosphorylation of phosphorylase *b* (Gross & Mayer, 1973). Our results confirm that troponin T is not phosphorylated at a significant rate by bovine 3':5'-cyclic AMP-dependent protein kinase. Likewise, examination of the data of Pratje & Heilmeyer (1972) indicates that, with a similar enzyme from skeletal muscle, only about 0.01 mol of P/mol of troponin T was incorporated after 60 min incubation at 30°C.

The marked inhibition of the phosphorylation of troponin T in the presence of troponin C suggests that interaction with troponin C either sterically blocks the phosphorylation sites or produces a conformational change in troponin T that renders the phosphorylation sites unavailable to the kinase. If the effect of the interaction with troponin C is simply to produce a steric blocking, these results would suggest that all three sites are fairly close together on the surface of troponin T. Interaction studies performed by gel electrophoresis indicate that a region of troponin T composed of the CNBr peptides CNB2 and CNB5 (Fig. 1) is involved in interaction with troponin C (P. Jackson, G. W. Amphlett & S. V. Perry, unpublished work). We consider that the inhibition of phosphorylation at sites II and III further supports the view that interaction with troponin C occurs in this region. This region is adjacent to, possibly overlapping, that part of the polypeptide chain which has been demonstrated to interact with tropomyosin (Fig. 1) (Jackson *et al.*, 1975).

The present study of the interaction of troponin C with troponin T reveals a number of features which resemble those observed in similar studies on troponin I (Perry & Cole, 1973; Perry *et al.*, 1976; Syska *et al.*, 1976). In both cases interaction with troponin C inhibits the phosphorylation of sites widely separated in the primary sequence. In troponin I and troponin T the phosphorylation sites appear to be either very close to, or possibly part of, the protein-protein interaction sites. These observations give further support to the view that a function of phosphorylation of the troponin complex is to alter the binding forces between the components by changing the net charge at the interaction sites and thus modulating the function of the complex.

Note Added in Proof (Received 8 December 1976)

The complete amino acid sequence of troponin T from white skeletal muscle has now been determined (Pearlstone *et al.*, 1976). From this sequence phosphorylation site I can be identified as serine-1, site II as serine-149 or -150 and site III as serine-156 or -157.

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