Biochem J. (1980) **191**, 547–554
Printed in Great Britain

Phosphorylation of rabbit cardiac-muscle troponin I by phosphorylase kinase

The effect of adrenaline

Arthur J. G. MOIR and S. Victor PERRY
Department of Biochemistry, University of Birmingham, P.O. Box 363, Birmingham B15 2TT, U.K.

(*Received 14 April 1980/Accepted 2 July 1980*)

1. Incubation of rabbit cardiac-muscle troponin I with phosphorylase *b* kinase leads to the incorporation of 0.7–1.2 mol of P_i/mol. 2. The major site of phosphorylation is a serine residue at position 72. 3. Lesser amounts of phosphate are incorporated into threonine-138, threonine-162 and serine-20. 4. Serine-20 is the only site that contains a significant amount of phosphate before incubation wtih phosphorylase *b* kinase. 5. Unlike the situation with serine-20, the extent of phosphorylation of serine-72 and threonine-138 in the perfused rabbit heart does not change when the heart is exposed to adrenaline (4 μM).

Studies on troponin I isolated from cardiac muscle have established that its phosphorylation can be catalysed by 3′:5′-cyclic AMP-dependent protein kinase and phosphorylase kinase (see Perry, 1979, for review). Phosphorylation by the former enzyme appears to be of special significance, as cardiac-muscle troponin I possesses an additional 26-residue sequence at the *N*-terminus that is absent from the skeletal-muscle forms of troponin I and that contains a serine residue at position 20 that is readily phosphorylated by 3′:5′-cyclic AMP-dependent protein kinase. In the perfused heart adrenaline causes an increase in the covalently bound phosphate of troponin I (England, 1975; Solaro *et al.*, 1976), which arises from phosphorylation at serine-20 (Moir *et al.*, 1980). This results in a decreased sensitivity of the actomyosin ATPase to Ca^{2+} and helps to stabilize the contractile response of the myocardium to β-adrenergic agonists and speed up relaxation (see Perry, 1979; Katz, 1979).

In our previous study (Moir *et al.*, 1980) it was noted that the phosphate content of cardiac-muscle troponin I isolated by affinity chromatography was greater than could be accounted for by the amount of phosphorylation at the sites known to be specific for 3′:5′-cyclic AMP-dependent protein kinase. This raises the possibility of the presence of phosphorylated residues on troponin I in addition to those specific to 3′:5′-cyclic AMP-dependent protein kinase. To investigate this aspect we have studied the sites of phosphorylation of cardiac-muscle troponin I that are specific for phosphorylase kinase. These sites may also be of significance in the interaction of cardiac-muscle troponin I with troponin C for, unlike the sites of phosphorylation specific for 3′:5′-cyclic AMP-dependent kinase, they are blocked in the presence of troponin C (Cole & Perry, 1975). This suggests that the sites of phosphorylation by phosphorylase kinase are rendered inaccessible to the enzyme either by direct steric blocking or by conformational changes induced in cardiac troponin I by the troponin C.

In the present paper serine-72 is identified as the major site of phosphorylation *in vitro* of rabbit cardiac-muscle troponin I by phosphorylase kinase. The inotropic effect of adrenaline on the perfused rabbit heart was not accompanied by significant phosphorylation at this site.

Materials and methods

Materials

Bovine cardiac 3′:5′-cyclic AMP-dependent protein kinase (type 1) was purchased from Sigma (London) Chemical Co., Poole, Dorset BN17 7NH, U.K. and trypsin (1-chloro-4-phenyl-3-L-tosylamidobutan-2-one-treated) was from Cambrian Chemicals, Croydon, Surrey CR0 4XB, U.K. [γ-^{32}P]ATP was supplied as the freeze-dried ammonium salt by The Radiochemical Centre, Amersham, Bucks., U.K. Polygram Ionex-25 SB-Ac/UV precoated t.l.c. plates were obtained from Camlab, Cambridge CB4 1TH, U.K. Frozen rabbit hearts were supplied by the Buxted Rabbit Co., Buxted, Sussex TN22 4LP, U.K.

0306-3275/80/110547-08$01.50/1

Preparation of troponin I

Troponin I was prepared from homogenates of frozen rabbit hearts in 9M-urea/75mM-Tris/15mM-β-mercaptoethanol/1mM-$CaCl_2$ (brought to pH 8.0 with 11.4M-HCl) by using a troponin C–Sepharose 4B affinity column as described by Syska *et al.* (1974). The same procedure was used to isolate the protein from rabbit hearts perfused as described by Moir *et al.* (1980) in the absence and presence of 4μM-adrenaline. The hearts were cut down at peak of force generation after perfusion with adrenaline and homogenized within 10–15s.

Phosphorylase kinase

This enzyme was prepared from rabbit skeletal muscle by the method of Cohen (1973).

Phosphorylation of troponin I

Rabbit cardiac-muscle troponin I (1–2mg/ml) was incubated at 30°C with phosphorylase *b* kinase (0.05–0.1mg/ml) in a solution containing 1–2 mM-[γ-^{32}P]ATP (5–7μCi/ml)/2.5mM-NaF/5.0mM-magnesium acetate/1.0mM-dithiothreitol/0.2mM-$CaCl_2$/50mM-sodium glycerophosphate/50mM-Tris, adjusted to pH 7.5 with 1M-HCl. After incubation for approx. 1h, the ^{32}P-labelled troponin I was desalted by gel filtration on Sephadex G-50 equilibrated against 10mM-HCl (Moir & Perry, 1977) and freeze-dried before digestion. Troponin I was phosphorylated by incubation with 3′:5′-cyclic AMP-dependent protein kinase under the conditions described by Moir & Perry (1977).

Identification of phosphorylated amino acids

Troponin I or peptides derived from troponin I (approx. 1nmol) were hydrolysed at 110°C for 2–4h in 5.7M-HCl and dried *in vacuo*. Hydrolysates were applied to precoated sheets (5cm × 20cm) of Polygram Ionex-25 SB-Ac/UV that had previously been washed by chromatography in formic acid/acetic acid/water (1:4:45, by vol.). The plates were developed by ascending chromatography in the same solvent, dried in air and the ^{32}P-labelled material was located by autoradiography. R_F values for phosphothreonine, phosphoserine and free orthophosphate were 0.49, 0.39 and 0.11 respectively. Alternatively, the hydrolysates were examined by paper electrophoresis at pH 2.0 (Moir *et al.*, 1980).

Isolation and identification of ^{32}P-labelled peptides

Peptides were obtained from cardiac-muscle troponin I by digestion of either the whole protein or CNBr peptides obtained from it with trypsin and purified by column chromatography followed by paper electrophoresis. ^{32}P-labelled peptides were located on chromatograms by autoradiography and were characterized by amino-acid analysis and partial sequence determination. The methods used for digestion with trypsin and CNBr, for separation of the CNBr peptides and for amino-acid analysis and sequence determinations, are described in detail by Moir & Perry (1977) and Moir *et al.* (1980). ^{32}P was determined by Čerenkov counting of aqueous solutions (Gould *et al.*, 1972).

Protein determination

Protein was measured by Nesslerization after digestion (Strauch, 1965), assuming that the protein contained 16% N.

Results

After incubation of rabbit cardiac-muscle troponin I with phosphorylase kinase in the presence of [γ-^{32}P]ATP (see the Materials and methods section) the ^{32}P content was 0.91 ± 0.14mol/mol of troponin I (mean ± S.E.M., $n = 4$). T.l.c. and paper electrophoresis of partial acid hydrolysates yielded radioactive spots that migrated with mobilities identical with those of phosphoserine and phosphothreonine.

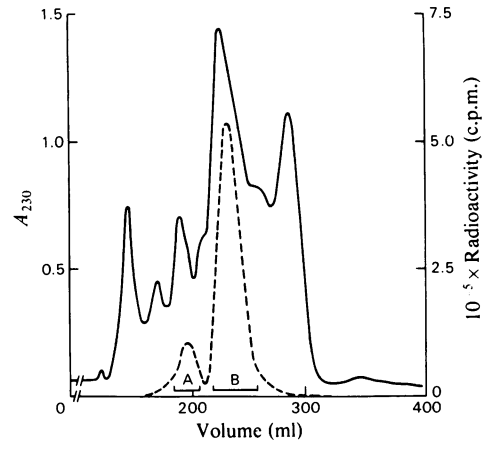

Fig. 1. *Gel filtration of the products of trypsin treatment of ^{32}P-labelled cardiac-muscle troponin I*

^{32}P-labelled cardiac-muscle troponin I (approx. 40mg) was digested with trypsin (Moir *et al.*, 1974) and applied to a column (2.5cm × 10cm) of Sephadex G-25 equilibrated and eluted with 0.01M-HCl. Fractions (5ml) were collected, the A_{215} measured, and applied to a column (2.5cm × 100cm) of Sep-Čerenkov procedure (Gould *et al.*, 1972). Fractions A and B, as represented by the bars, containing ^{32}P were pooled. ——, A_{230}; – – –, radioactivity.

Identification of ^{32}P-labelled peptides obtained from cardiac-muscle troponin I digested wtih trypsin

When the products of ^{32}P-labelled cardiac-muscle troponin I digestion with trypsin were subjected to gel filtration on Sephadex G-25, two peaks of radioactivity were present in the eluate. These were pooled and named fractions A and B (Fig. 1). Fraction B contained 85% of the total peptide-bound ^{32}P and separated into about nine radioactive bands on electrophoresis at pH 6.5 (Fig. 2*a*). By further electrophoresis at pH 2.0 and 3.5, three of these peptides, designated BD, BE and BF (Fig. 2*a*), which together accounted for 75% of the total radioactivity in fraction B, were obtained in sufficient yield to allow positive identification. Their amino-acid and ^{32}P contents are given in Table 1.

Peptide BF contained 71% of the ^{32}P recovered from fraction B associated with the three major radioactive peptides and therefore contained the main site of phosphorylation by phosphorylase kinase. From the *N*-terminal sequence determination

Table 1. *Amino-acid compositions and ^{32}P contents of peptides isolated from a digestion by trypsin of cardiac-muscle troponin I*

Radioactive peptides were isolated from fractions A and B (see Fig. 1) from a digestion by trypsin of rabbit cardiac-muscle troponin I. Amounts are expressed as mol of residue or ^{32}P/mol of peptide. –, Designates residue absent or present at less than 0.25 mol/mol.

	Content (mol/mol of peptide)			
Peptide ...	BF	BD	BE	AC
Asp	–	–	0.9	1.3
Thr	0.9	1.1	–	0.6
Ser	1.0	–	1.2	–
Glu	–	–	–	1.4
Pro	–	1.4	–	–
Ala	1.2	–	0.9	0.8
Leu	1.1	1.9	–	2.0
Tyr	–	–	0.8	–
Lys	–	–	–	1.0
Arg	0.9	1.8	2.1	0.9
^{32}P	0.97	1.23	0.03	0.91

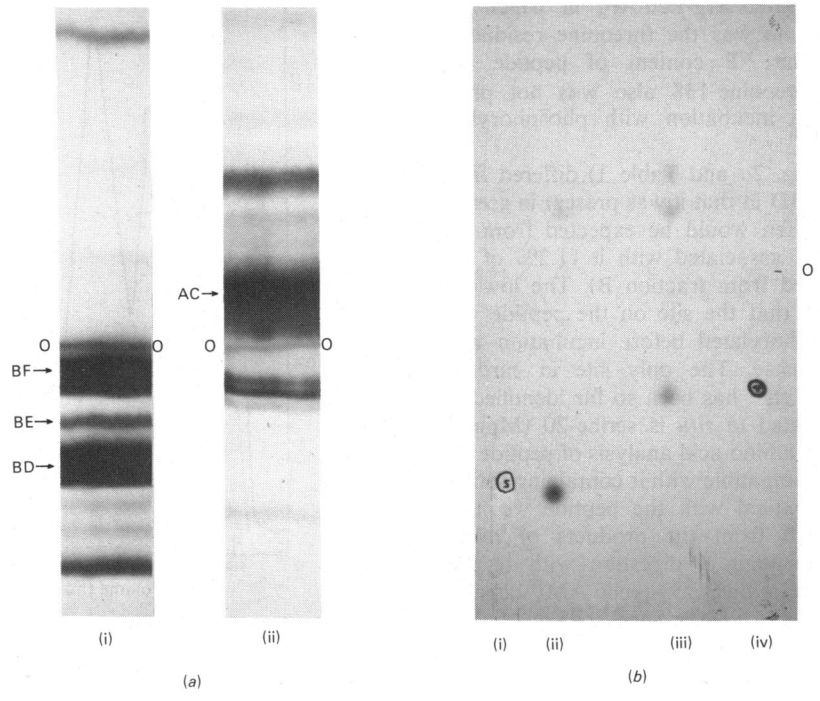

Fig. 2. *Autoradiography of peptides and phosphorylated amino acids from digests of ^{32}P-labelled cardiac-muscle troponin I*

(*a*) Autoradiographs of paper electrophoretograms carried out at pH 6.5 on pooled fractions obtained by gel filtration of a ^{32}P-labelled rabbit cardiac-muscle troponin I digested with trypsin (see Fig. 1). O, origin. Peptides described in the text are indicated by arrows. (i) Peak B; (ii) peak A. (*b*) Autoradiographs of electrophoretograms carried out at pH 2.0 of samples of peptides hydrolysed in 5.7M-HCl at 110°C for approx. 2h. Authentic samples of serine phosphate and threonine phosphate were located by staining with ninhydrin and the spots were ringed with radioactive ink. (i) Serine phosphate; (ii) hydrolysate of peptide BF; (iii) hydrolysate of peptide BD; (iv) threonine phosphate.

and the amino-acid analysis data (Table 1) the provisional structure Ala-Leu(Thr,Ser,Arg) was given to the peptide. In view of the known sequence of cardiac-muscle troponin I (Grand *et al.*, 1976), the only possible conclusion was that the peptide represented the region consisting of residues 70–74 and that the complete sequence was Ala-Leu-Ser-Thr-Arg. Partial acid hydrolysis of peptide BF indicated the presence of phosphoserine, but not of phosphothreonine (Fig. 2*b*). It follows therefore that serine-72 was the phosphorylation site in peptide BF and since the ^{32}P content of the peptide was 0.97 mol/mol of peptide this residue was not significantly phosphorylated in cardiac-muscle troponin I before incubation.

Peptide BD (Fig. 2*a* and Table 1) accounted for 26% of the ^{32}P recovered from fraction B as the three peptides. Amino-acid analysis indicated that it was a hexapeptide containing one threonine residue and partial acid hydrolysis of peptide BD confirmed the presence of phosphothreonine. From comparisons of the amino-acid composition with the sequence of cardiac-muscle troponin I it was concluded that the peptide was derived from the sequence Pro-Thr-Leu-Arg-Leu-Arg in which the phosphorylation site was the threonine residue at position 138. The ^{32}P content of peptide BD indicated that threonine-138 also was not phosphorylated before incubation with phosphorylase kinase.

Peptide BE (Fig. 2*a* and Table 1) differed from peptides BF and BD in that it was present in greater molar amounts than would be expected from the proportion of ^{32}P associated with it (1.2% of the total ^{32}P recovered from fraction B). The low ^{32}P content indicated that the site on the peptide was extensively phosphorylated before incubation with phosphorylase kinase. The only site in cardiac-muscle troponin I that has been so far identified as being phosphorylated *in situ* is serine-20 (Moir & Perry, 1977). The amino-acid analysis of peptide BE was completely compatible with it containing serine-20 and being identical with the peptide we have previously isolated from the products of rabbit cardiac-muscle troponin I digestion with trypsin after phosphorylation by 3′:5′-cyclic AMP-dependent protein kinase, i.e. Arg-Ser-Asp-Arg-Ala-Tyr (Moir & Perry, 1977).

Rabbit cardiac-muscle troponin I contains tyrosine residues at positions 24 and 107. The amino-acid composition of peptide BE is such that it can only be derived from the region around tyrosine-24. The *N*-terminal amino acid of peptide BE was arginine, but no further sequence data were obtained. Edman degradation of peptides BE and BF did not release the expected phosphoserine residue or subsequent residues.

Peptide BE is an unusual tryptic peptide in that it contains a *C*-terminal tyrosine residue and two residues of arginine that are not cleaved during digestion with trypsin. Cleavage at tyrosine by trypsin is not uncommon and may result from the autocatalytic formation of pseudotrypsin (Smith & Shaw, 1969). We have reported (Moir & Perry, 1977; Moir *et al.*, 1980) limited cleavage of the Arg-Ser*P* bond in the sequence Arg-Ser*P*-Asp-Arg-Ala-Tyr, but have never observed cleavage of the Arg-Ala bond after phosphorylation of serine-20. It has been reported (Schroeder, 1968) that the rate of cleavage at arginine by trypsin is greatly decreased by the presence of a residue of aspartic acid at the *N*-terminal side. The sequence Ser*P*-Asp-Arg-Ala might thus be expected to be cleaved at a very low rate by trypsin. If the serine remains unphosphorylated, some cleavage at the arginine residue does occur.

Fraction A, the minor fraction of ^{32}P-labelled

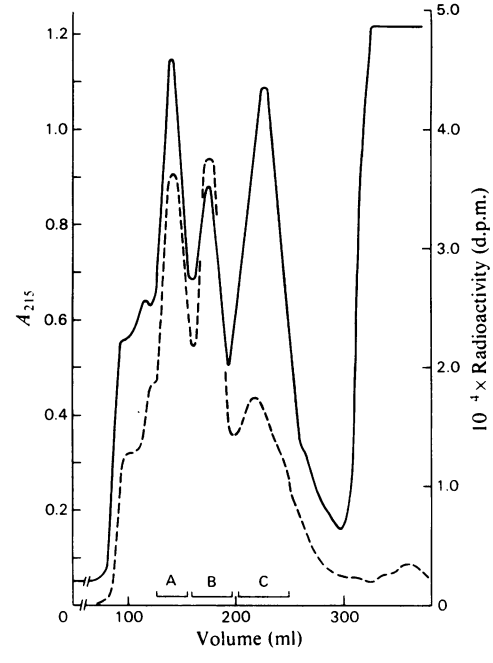

Fig. 3. *Gel filtration of ^{32}P-labelled cardiac-muscle troponin I digested with CNBr*

^{32}P-labelled cardiac-muscle troponin I (20 mg) was digested with CNBr in 0.1 M-HCl and carboxymethylated (Moir *et al.*, 1974). The digest was applied to a column (2.5 cm × 110 cm) of Sephadex G-75, equilibrated and eluted with 0.01 M-HCl. Fractions (5 ml) were collected, the A_{215} was measured and ^{32}P radioactivity was determined by the Čerenkov procedure. The peptides from CNBr treatment were pooled in fractions A, B and C as indicated by the bars. ——, A_{215}; – – – –, radioactivity.

peptides, obtained on gel filtration of digests from trypsin treatment was fractionated further by electrophoresis at pH6.5 (Fig. 2*a*). Only one of the ^{32}P-containing peptides, peptide AC (Table 1), representing 37% of the total recovered ^{32}P in fraction A, was obtained in sufficient yield to allow positive identification from its amino-acid composition. This indicated that peptide AC was derived from the sequence Ala-Lys-Glu-Thr-Leu-Asp-Leu-Arg. It was therefore concluded that threonine-162 was the site of phosphorylation and judging from the ^{32}P content of the peptide this residue was not significantly phosphorylated before incubation with phosphorylase kinase.

Identification of ^{32}P-labelled peptides from trypsin treatment of CNBr peptides of cardiac-muscle troponin I

Cardiac-muscle troponin I that had previously been phosphorylated with phosphorylase kinase in the presence of [γ-^{32}P]ATP was digested with CNBr in 0.1M-HCl and the digest was fractionated on Sephadex G-75 (Moir & Perry, 1977; Moir *et al.*, 1980). The major phosphorylated peptides (Fig. 3) were peptides CCN2 (residues 49–150, peak B) and CCN1 (residues 1–150, peak A). A small proportion of radioactivity (23% of total) was also present in the peak (peak C) containing peptides CCN3 (residues 1–48) and CCN4 (residues 152–197).

The radioactive peptides obtained by trypsin treatment of peptide CCN2 were purified by gel filtration, followed by paper electrophoresis at pH6.5, 3.5 and 2.0. In all, about eight different radioactive peptides could be separated, but most contained only a small fraction of the recovered radioactivity. Two radioactive peptides were isolated in adequate yields for characterization by amino-acid analysis. The major phosphorylated peptide from trypsin treatment, which contained 62% of the recovered radioactivity, had an amino-acid composition and electrophoretic mobilities identical with those of peptide BF. A further 19% of the recovered radioactivity was associated with a peptide that had the same electrophoretic mobilities and amino-acid composition as peptide BD.

In view of these facts it was concluded that the major phosphorylated peptides in these digests were peptides identical with peptides BF and BD obtained by direct tryptic digestion of cardiac troponin I.

Tryptic digestion of the fraction containing peptides CCN3 and CCN4 produced four ^{32}P-containing peptides. Only one, identical with peptide BE described above could be obtained in sufficient yield to allow positive characterization by amino-acid analysis and electrophoretic behaviour. A peptide corresponding to the minor phosphorylated peptide AC obtained from trypsin treatment of whole troponin I (see above) was not identified after CNBr digestion of ^{32}P-labelled cardiac-muscle troponin I (two experiments).

Phosphorylation of troponin I prepared from the rabbit heart perfused in the presence and absence of adrenaline

Samples of troponin I prepared from control and adrenaline-stimulated perfused rabbit hearts (Moir *et al.*, 1980) were incubated in the presence of [γ-^{32}P]ATP with phosphorylase *b* kinase and 3′:5′-cyclic AMP-dependent protein kinase. On incubation with phosphorylase kinase the total amounts of ^{32}P incorporated into troponin I from control and adrenaline-stimulated hearts (Table 2) were very similar. As might be expected from this result there was little difference in the distribution of the ^{32}P-labelled peptides when products from digestion with CNBr of the two phosphorylated samples were compared by gel filtration on Sephadex G-75. In both cases radioactivity was present in peptides CCN1, CCN2, CCN3 and CCN4 (Fig. 4).

If the troponin I prepared from the control and adrenaline-treated hearts was incubated under similar conditions with 3′:5′-cyclic AMP-dependent kinase a significant difference was observed

Table 2. *Phosphorylation of troponin I prepared from control and adrenaline-stimulated perfused rabbit hearts*
Troponin I was isolated by affinity chromatography and incubated with [γ-^{32}P]ATP and enzymes as indicated in the Materials and methods section.

Enzyme	Time of incubation (min)	$10^{-2} \times$ ^{32}P incorporated (c.p.m.) Control (A)	Stimulated (B)	(A)/(B)
(*a*) Protein kinase	5	1300	506	2.6
	10	6900	1630	4.2
	20	5126	3182	1.6
	45	1038	323	3.2
(*b*) Phosphorylase kinase	120	5738	5689	1.0
	120	2248	2301	0.97

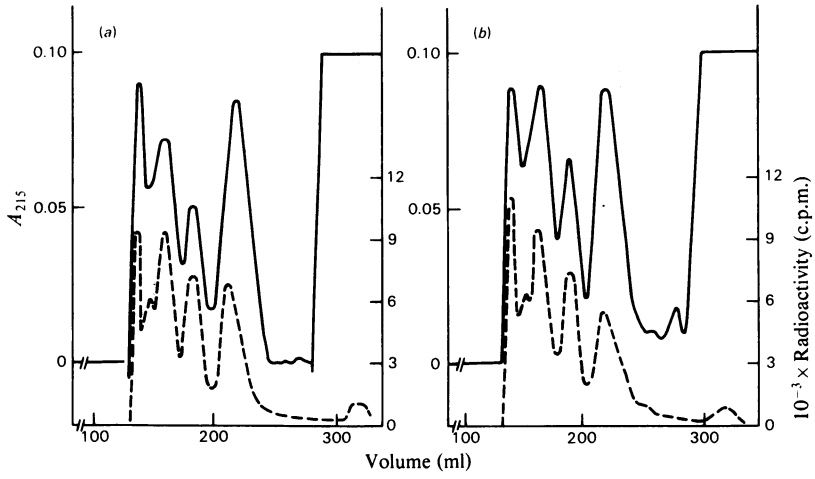

Fig. 4. *Gel filtration of the products of CNBr treatment of troponin I isolated from perfused rabbit hearts and incubated with phosphorylase kinase*

Cardiac-muscle troponin I (approx. 3 mg) prepared from control and adrenaline-stimulated hearts was incubated under the conditions described in the Materials and methods section with [γ-^{32}P]ATP and phosphorylase *b* kinase, digested with CNBr and the digest fractionated as described in the legend to Fig. 3. (*a*) Troponin I from control hearts; (*b*) troponin I from hearts stimulated with adrenaline (4 μM). ——, A_{215}; – – – –, radioactivity.

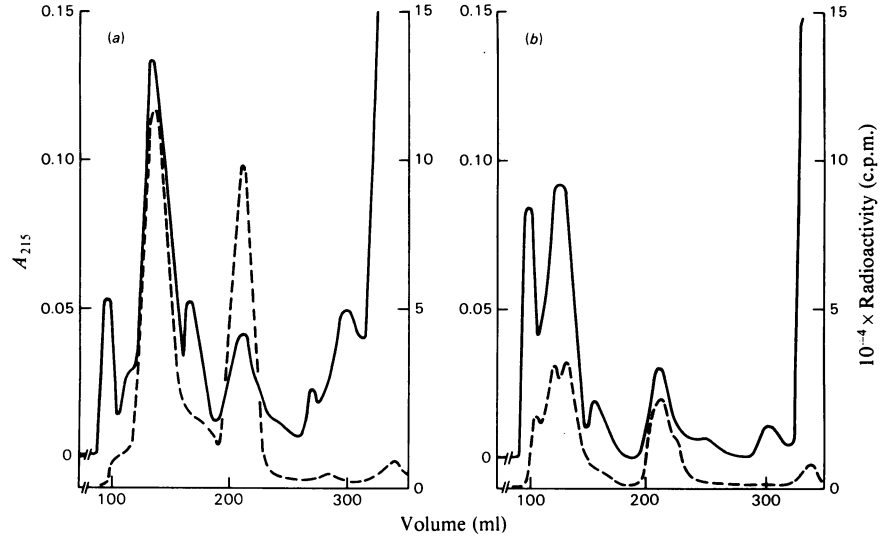

Fig. 5. *Gel filtration of the products of CNBr treatment of troponin I isolated from perfused rabbit hearts and incubated with 3′:5′-cyclic AMP-dependent protein kinase*

Cardiac-muscle troponin I (approx. 3 mg) prepared from control and adrenaline-stimulated hearts was incubated under the conditions described in the Materials and methods section with [γ-^{32}P]ATP and cyclic AMP-dependent protein kinase, digested with CNBr and the digest fractionated as described in the legend to Fig. 3. (*a*) Troponin I from control hearts; (*b*) troponin I from hearts stimulated with adrenaline (4 μM). ——, A_{215}; – – – –, radioactivity.

between the two samples of troponin I. In both cases radioactivity was confined to peptides CCN1 and CCN3 from CNBr treatment (Fig. 5), but the extent of labelling was much decreased in troponin I isolated from adrenaline-treated hearts. This correlated well with the observation that the total ^{32}P incorporated in the latter troponin I was much less than in the troponin I isolated from the control hearts (Table 2).

Discussion

On incubation with phosphorylase *b* kinase, phosphate was incorporated into four sites on cardiac-muscle troponin I, serine-72, threonine-138, threonine-162 and serine-20 (Table 3 and Fig. 6). Neither of the two major sites of phosphorylation, serine-72 and threonine-138, correspond to the major site of phosphorylation of fast-skeletal-muscle troponin I by this enzyme. This latter site, threonine-11, in the sequence Arg-Ile-Thr-Ala-Arg (Table 3) is equivalent to serine-37 in cardiac-muscle troponin I. Although the sequence around the cardiac-muscle troponin I site is similar to that around threonine-11 in fast-skeletal-muscle troponin I, serine-37 is presumably not recognised as a phosphorylation site by phosphorylase kinase because the requirement for the two residues on the carboxy side of the phosphorylation site to be successively hydrophobic and charged (arginine) is not fulfilled.

Serine-146 is the equivalent residue on cardiac-muscle troponin I to serine-117 in fast-skeletal-muscle troponin I, which is a minor site of phosphorylation catalysed by phosphorylase kinase (Fig. 6). In the present study serine-146 was not identified as a significant site, but may have been located on one of the minor radioactive peptides present in amounts that were too small for identification. In this respect it is of interest that serine-117 of fast-skeletal-muscle troponin I and serine-146 of cardiac-muscle troponin I are both major phosphorylation sites for cyclic AMP-dependent protein kinase.

The sequence around serine-72, the major phosphorylation site, is very similar to that at the phosphorylation site of phosphorylase, whereas that around threonine-138 is somewhat less similar on the amino side, but maintains the 'hydrophobic residue-Arg' sequence on the carboxy side. The sequences on the carboxy sides of the other phosphorylated residues do not maintain this similarity, although they do contain arginine or aspartic acid in the second position. The differences in the extent of phosphorylation of each of the four sites in cardiac-muscle troponin I presumably reflect the extent to which the sequences resemble the sequence around the phosphorylation site of phosphorylase.

When troponin C is present in equimolar or greater proportions, the phosphorylation of cardiac-

Table 3. *Comparison of amino-acid sequences adjacent to residues phosphorylated by phosphorylase kinase in phosphorylase, cardiac-muscle troponin I and fast-skeletal-muscle troponin I from the rabbit*

Source	Sequence
Phosphorylase	Gln-Ile-Ser*P*-Val-Arg*
Cardiac-muscle troponin I (Ser-72)	Ala-Leu-Ser*P*-Thr-Arg
Cardiac-muscle troponin I (Thr-138)	Arg-Pro-Thr*P*-Leu-Arg
Cardiac-muscle troponin I (Ser-20)	Arg-Arg-Ser*P*-Asp-Arg
Cardiac-muscle troponin I (Thr-162)	Lys-Glu-Thr*P*-Leu-Asp
Skeletal-muscle troponin I (Thr-11)	Arg-Ile-Thr*P*-Ala-Arg†‡
Skeletal-muscle troponin I (Ser-117)	Arg-Met-Ser*P*-Ala-Asp†‡
Skeletal-muscle troponin I (Ser-89)	Lys-Ser-Ser*P*-Lys-Glu‡

* Nolan *et al.* (1964). † Moir *et al.* (1974). ‡ Huang *et al.* (1974).

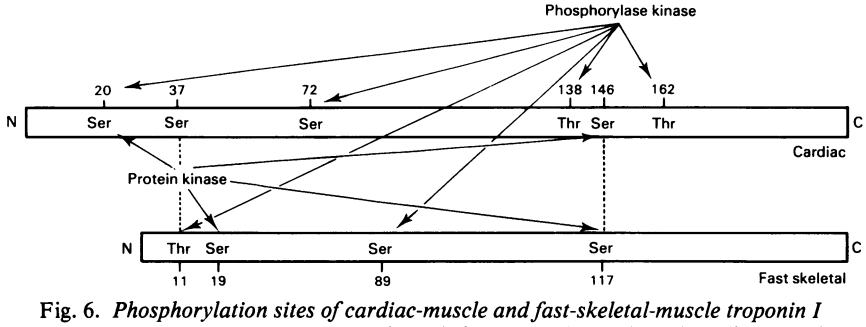

Fig. 6. *Phosphorylation sites of cardiac-muscle and fast-skeletal-muscle troponin I*
Schematic representation of the primary sequences of rabbit fast-skeletal-muscle and cardiac-muscle troponin I. The arrows indicate the enzymes that phosphorylate the sites the positions of which in the sequences are represented by numbers. N, *N*-terminus; C, *C*-terminus.

muscle troponin I by phosphorylase kinase is inhibited by 90% (Cole & Perry, 1975). The major sites of phosphorylation, serine-72 and threonine-138, must therefore be rendered inaccessible to phosphorylase kinase when troponin C is present. Such inhibition is usually explained by direct steric blocking of the sites by troponin C and the studies with isolated fragments of skeletal-muscle troponin I would tend to support this view (Syska *et al.*, 1976; Perry, 1979). This would be expected in the case of threonine-138, which lies within the region of the actin-binding site of cardiac-muscle troponin I and therefore is very likely to be unavailable for phosphorylation when the inhibitory action of troponin I is neutralized by troponin C. Other studies on skeletal-muscle troponin I (Cole & Perry, 1975) suggest that the actin-binding site is either close to or part of a site presumed to be a site of interaction with troponin C. There is also evidence for troponin C interaction at the *N*-terminal region in fast-skeletal-muscle troponin I, which is equivalent to residues 27–46 of cardiac-muscle troponin I (Grand *et al.*, 1976). Since phosphorylation of serine-20 of cardiac-muscle troponin I remains unaffected when cyclic AMP-dependent kinase is the phosphorylating enzyme (Moir & Perry, 1977), the present results would suggest that troponin C interaction with the *N*-terminal end of cardiac-muscle troponin I does not involve serine-20 but extends up to residue-72 at least. Phosphorylation of serine-20 is probably responsible for the 10–20% phosphorylation of cardiac-muscle troponin I catalysed by phosphorylase kinase in the presence of troponin C.

Although phosphorylation of cardiac-muscle troponin I by phosphorylase kinase can occur *in vitro*, the sites specific for the enzyme (serine-72, threonine-138 and threonine-162) are not normally phosphorylated in the functioning perfused heart. This was suggested by previous studies (Moir *et al.*, 1980) and the present investigation confirms that this is the case in both the control unstimulated heart and the heart perfused in the presence of adrenaline. Thus it would appear that the phosphorylase kinase-catalysed phosphorylation of serine-72, threonine-138 and threonine-168 is not involved in the changes in phosphorylation of troponin I that are associated with the inotropic response to adrenaline. From studies on perfused hearts from phosphorylase kinase-deficient (I strain) mice, England (1977) has concluded that phosphorylase kinase does not phosphorylate troponin I. The studies *in vitro* suggest, however, that phosphorylation of serine-20 may be catalysed to a minor extent by phosphorylase kinase as well as by cyclic AMP-dependent protein kinase during the inotropic response to adrenaline.

We thank Miss Sue Brewer for carrying out the amino-acid analyses. This work was supported in part by a grant from the Medical Research Council.

References

Cohen, P. (1973) *Eur. J. Biochem.* **34**, 1–14
Cole, H. A. & Perry, S. V. (1975) *Biochem. J.* **149**, 525–533
England, P. J. (1975) *FEBS Lett.* **50**, 57–60
England, P. J. (1977) *Biochem. J.* **168**, 307–310
Gould, J. M., Cather, R. & Winget, G. D. (1972) *Anal. Biochem.* **50**, 540–548
Grand, R. J. A., Wilkinson, J. M. & Mole, L. E. (1976) *Biochem. J.* **159**, 633–641
Huang, T. S., Bylund, D. B., Stull, J. T. & Krebs, E. G. (1974) *FEBS Lett.* **42**, 249–252
Katz, A. M. (1979) *Adv. Cyclic Nucleotide Res.* **11**, 303–343
Moir, A. J. G. & Perry, S. V. (1977) *Biochem. J.* **167**, 333–343
Moir, A. J. G., Wilkinson, J. M. & Perry, S. V. (1974) *FEBS Lett.* **42**, 253–256
Moir, A. J. G., Solaro, R. J. & Perry, S. V. (1980) *Biochem. J.* **185**, 505–513
Nolan, C., Novoa, W. B., Krebs, E. G. & Fischer, E. H. (1964) *Biochemistry* **3**, 542–551
Perry, S. V. (1979) *Biochem. Soc. Trans.* **7**, 593–617
Schroeder, W. A. (1968) *The Primary Structure of Proteins*, Harper and Row, New York and London
Smith, R. L. & Shaw, E. (1969) *J. Biol. Chem.* **244**, 4704–4712
Solaro, R. J., Moir, A. J. G. & Perry, S. V. (1976) *Nature (London)* **262**, 615–617
Strauch, L. (1965) *Z. Klin. Chem.* **3**, 165–167
Syska, H., Perry, S. V. & Trayer, I. P. (1974) *FEBS Lett.* **40**, 253–257
Syska, H., Wilkinson, J. M., Grand, R. J. A. & Perry, S. V. (1976) *Biochem. J.* **153**, 375–387